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PCT #5

I hereby certify that this correspo[illegible] and a copy of each document referenced herein is being depos[illegible] the United States Postal Service in an envelope addressed to Commissi[illegible]or Patents, Alexandria, VA 22313-1450 utilizing the "Express Mail Post Office to Addressee" service under Mailing Label No. EV 532466605 US, on the date shown below.
Dated: July 20, 2005 Signature: (Richard Zimmermann)

JC10 Rec'd PCT/PTO 20 JUL 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Muntau-Heger et al.

Serial No.: 10/539,842 (U.S. national phase of Int'l Application No. PCT/EP2003/014262)

Int'l filing date: December 15, 2003
Priority date: December 20, 2002

Art Unit: Not Yet Assigned

For: Use of Tetrahydrobiopterin Derivatives in the Treatment and Nutrition of Patients with Amino Acid Metabolic Disorders.

Examiner: Not Yet Assigned

TRANSMITTAL LETTER WITH PROTEST AND EXHIBITS AA-X

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Enclosed is a Protest Pursuant to 37 C.F.R. §1.291, with a Certificate of Service, and a copy of each of the documents listed below:

AA. A certified English translation of PCT/EP2003/014262

A. **Bates et al.,** *Medication Administration: The Correct Way*, in Children's Medications- A Parent's Guide, Harvey Whitney Books Company (1996)

B. **Blau et al.**, 34th EMG Meeting, Zurich, CH, held May 31-June 2, 2002, proceedings published October 2002

C. **Curtius et al.**, *The Lancet*, 1:657-658 (1983)

D. **Curtius et al.**, *Advances in Neurol.* 40:463-466 (1984)

E. **Curtius et al.,** U.S. Patent No. 4,774,244

F. **Dissing et al.**, *Acta Neurol. Scand.* 79:493-99 (1989)

G. **Ellis**, *Philos Trans R Soc Lond B Biol Sci.*, 339:257-61 (1993)

H. **Erlandsen et al.**, *J Inherit Metab Dis* 24:213-30 (2001)

I. **Hansen et al.**, U.S. Patent No. 5,763,392

J. **Hennermann et al.**, Society for the Study of Inborn Errors of Metabolism 40th Annual Symposium. Dublin, Ireland, 3-6 Sept. 2002, abstract published in *J Inherit Metab Dis.*, Suppl 1:1-184, July 2002

K. **Isaacs**, *American Druggist* 216(6):37-41 (1999)

L. **Kure et al.**, *J Pediatrics* 135:375-8, 1999

M. **Mukerji et al.**, U.S. Patent No. 6,428,990

N. **Nagatsu,** Essays in Biochemistry, 30:15-35 (1995)

O. **Naruse et al.**, U.S. Patent No. 4,778,794

P. **PhenylAde Amino Acid Bars** brochure (hereinafter "**Phenylade I brochure**") dated November 2002

Q. **PhenylAde Amino Acid Blends** brochure (hereinafter "**Phenylade II brochure**") dated May 2002

R. **Rabelink et al., US Patent Application No. 2002/0052374**, published May 2002

S. **Schaub et al.**, Arch Dis Child. 53(8):674-6 (1978)

T. **Shinozaki et al.**, *Circ Res.* 87:566-73 (2000)

U. **Steinfeld et al.**, *Eur J Pediatr.* 161:403-405 (May 2002)

V. **Trefz et al.**, *Eur. J Pediatr.* 160:315, 2001

W. **Walter et al.**, *Am J Respir Crit Care Med.* 156(6):2006-10 (1997)

X. **Weglage et al.**, *J. Inherit. Metab. Dis.* 25 321-322 (2002)

Dated: July 20, 2005

Respectfully submitted,

By Li Hsien Rin Laures

Li-Hsien Rin-Laures, M.D.
Registration No.: 33,547
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive, Suite 6300
Sears Tower
Chicago, Illinois 60606-6357
(312) 474-6300

I hereby certify that this correspondence is being deposited with the United States Postal Service in an envelope addressed to Commissioner for Patents, Alexandria, VA 22313-1450 utilizing the "Express Mail Post Office to Addressee" service under mailing Label No. EV 532466605 US on the date shown below.

Dated: July 20, 2005 Signature: (Richard Zimmermann)

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Examiner: Not Yet Assigned

PROTEST PURSUANT TO 37 CFR §1.291

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This protest of U.S. Patent Application No. 10/539,842 (U.S. national phase of Int'l Application No. PCT/EP2003/014262) pursuant to 37 CFR §1.291(a) is being submitted before the publication of the U.S. national phase application.

A copy of this protest has been served upon the applicant pursuant to 37 CFR § 1.248.

I. INTRODUCTION

The following protest is filed against U.S. Patent Application No. 10/539,842 (U.S. national phase of Int'l Application No. PCT/EP2003/014262) of Muntau-Heger et al., filed December 15, 2003 (the "Muntau-Heger application"). Pursuant to 37 C.F.R. §1.291, this paper includes (1) a listing of the publications relied upon, all of which were published more than one year before the December 15, 2003 filing date of the Muntau-Heger application, (2) a concise explanation of the relevance of each listed item; and (3) a copy of each listed publication (each of which is in the English language).

Claims are described herein with reference to the claims of Int'l Appl. No. PCT/EP2003/014262 as published in WO 2004/058268. A copy of an English language translation of WO 2004/058268 is supplied herewith.

II. DOCUMENTS RELIED UPON

Bates et al., *Medication Administration: The Correct Way*, in Children's Medications- A Parent's Guide, Harvey Whitney Books Company (1996) (hereinafter "**Bates**"). Bates discloses that medications can be administered by mixing them in beverages or foods. Medications can be combined with a number of foods including fruit juice, ice cream or soft food (Bates, page 6).

Blau et al., 34th EMG Meeting, Zurich, CH, held May 31-June 2, 2002, proceedings published October 2002 (hereinafter "**Blau**"). Blau discloses oral administration of BH4 to patients aged 4 to 14 having mild PKU, with doses of BH4 given orally over a range of 7.1-10.7 mg/kg (p.21). Blau further discloses that the mechanism underlying BH4 mediated affects on PKU patients may be through compensation for a reduced affinity of the PAH enzyme for BH4, by stabilization of the protein, by induction of PAH gene expression, and/or by introduction of 3D structural changes in the PAH protein (p. 19), which are properties of a chaperone molecule or messenger enhancer.

Curtius et al., *The Lancet*, 1:657-658 (1983). (hereinafter "**Curtius**"). Curtius reports that treatment of depressed patients with BH4 results in mood improvement and states

that "High-dose BH4 treatment has also been shown to be effective in certain cases of BH4-deficient hyperphenylalaninaemia, Parkinson's disease, and dystonia." Page 658, 1st col. The depressed patient was treated concurrently with BH4 and with the amino acids tryptophan and tyrosine. Page 658, 1st col.

Curtius et al., *Advances in Neurol.* 40:463-466 (1984) (hereinafter "**Curtius II**"). Curtius II reports that treatment of patients suffering from Parkinson's disease with BH4 (tetrahydrobiopterin dihydrochloride from Dr. B. Schirks) provided beneficial clinical effects. Page 464. BH4 dihydrochloride was administered (page 464). Curtius II also states that the aim of BH4 administration in Parkinson's disease is to elevate the activity of existing tyrosine hydroxylase molecules above whatever activity is provided by endogenous BH4 (page 465). Curtius II further states that BH4 is thought to play an important role in regulating the *in vivo* activities of tyrosine hydroxylase and tryptophan hydroxylase, which are rate-limiting enzymes in the synthesis of the catecholamines and serotonin, respectively (page 463).

Curtius et al., U.S. Patent No. 4,774,244 (hereinafter "**Curtius III**"). Curtius III discloses treatment of patients suffering from Parkinson's disease and patients suffering from depression with any one of L-erythro-5,6,7,8-tetrahydrobiopterin (BH4), L-sepiapterin, 1',2'-diacetyl-5,6,7,8-tetrahydrobiopterin or 6-methyl-5,6,7,8-tetrahydrobiopterin. Col. 1, lines 18-24. The BH4 was mixed with orange juice and administered to patients orally. Col. 1, lines 62-64. Patients' symptoms improved after administration of BH4. Cols. 2-3.

Dissing et al., *Acta Neurol. Scand.* 79:493-99 (1989) (hereinafter "**Dissing**"). Dissing discloses that individuals deficient in BH4 demonstrate defects in biosynthesis of neurotransmitters such as dopamine (a catecholamine) and norepinephrine. P. 493. Treatment of Parkinson's disease involves administration of neurotransmitter precursors such as L-dopa (page 494). Dissing describes the administration of BH4, either alone or in conjunction with the amino acid tyrosine, to patients having Parkinson's disease (page 495), indicting that BH4 is useful as a neurotransmitter similar to L-dopa.

Ellis, *Philos Trans R Soc Lond B Biol Sci.*, 339:257-61 (1993) (hereinafter "**Ellis**") discusses the role of molecular chaperones generally. Ellis states that molecular chaperones are defined in functional terms as a class of protein that assist the correct non-covalent assembly of other proteins in vivo, including assisting in correct folding of such

proteins. Page 258, col.2. They appear to act by binding to interactive protein surfaces that are transiently exposed during various cellular processes (page 259, col. 1); this binding inhibits incorrect interactions that may otherwise produce non-functional structures. Page 259, col. 2.

Erlandsen et al., *J Inherit Metab Dis* 24:213-30 (2001) (hereinafter "**Erlandsen**"). Erlandsen describes that PKU patients suffer from elevated levels of phenylalanine, most often attributed to impaired function of the enzyme PAH, caused by mutations in the gene encoding PAH (page 214). Patients often are treated by lifelong dietary restriction of phenylalanine intake (page 214). Erlandsen further describes that several PAH mutations have been identified and that particular mutations correlate with disease severity (pages 215, 222). Expression of these mutant PAH enzymes affects PAH enzymatic activity and dietary tolerance of phenylalanine (page 215). Erlandsen concludes that oral administration of BH4 allows the mutant PAH enzymes to overcome the lowered binding of BH4 to the PAH enzyme, thereby reducing blood phenylalanine levels to safer levels. Erlandsen indicates that patients taking oral BH4 supplements thus have a greater protein tolerance and may avoid treatment with a low-phenylalanine, low protein diet (page 227).

Hansen et al., U.S. Patent No. 5,763,392 (hereafter "**Hansen**"). Hansen, in the background section, states that myo-inositol is frequently added to infant formulas and adult nutritional formulas. Col. 1, lines 38-40. Hansen describes the administration of higher levels of myo-inositol to diabetics in order to lower plasma glucose levels. Col. 2, lines 5-7. Hansen teaches that such therapeutic levels of myo-inositol can be administered in any form, e.g. tablet, capsule, powder, suspension or solution. Col. 3, lines 47-48. Hansen also teaches that a preferred means of administering such medication is to incorporate it into a nutritional composition that is consumed by the subject. Col. 3, lines 47-51. Exemplary infant and adult nutritional compositions are described that include protein, lipid and carbohydrate. Col. 3, line 64 to col. 4, line 2. The protein component may be 7-30% of the total caloric value of the composition, including lower protein amounts such as 10-15% or 12%. Col. 4, lines 9-21. A number of carbohydrate sources are listed, including glucose, maltodextrin, and various starches. Col. 4, lines 32-46. A number of lipid sources are listed, including safflower oil, coconut oil, soybean oil, fish oil, and omega 3 and omega 6 fatty acids. Col. 4, lines 47- col. 5, line 1. A number of minerals and vitamins may be included, including choline. Col. 5,

lines 22-29. The composition may also be flavored, including chocolate, coconut, banana or strawberry flavored. Col. 5, lines 45-47.

Hennermann et al., Society for the Study of Inborn Errors of Metabolism 40th Annual Symposium. Dublin, Ireland, 3-6 Sept. 2002, abstract published in *J Inherit Metab Dis.*, Suppl 1:1-184, July 2002 (hereinafter "**Hennermann**"). Hennermann describes treatment of patients with both mild and classic PKU by daily administration of BH4 over a prolonged period of time (e.g., 4-13 months). Hennerman reports successful treatment of both mild and classic PKU with BH4, with an increase of the daily phenylalanine tolerance of 180-1830 mg (line 13). Hennerman indicates that patients with classic PKU need to maintain a low phenylalanine diet.

Isaacs, *American Druggist* 216(6):37-41 (1999) (hereinafter "**Isaacs**"). Isaacs discloses that medications can be administered by mixing them in formula or foods (page 38, 3rd col.). Medications can be combined with a number of foods including rice formula, cereal, pudding applesauce, mashed potatoes, yogurt, or jello (page 38, 3rd col.).

Kure et al., *J Pediatrics* 135:375-8, 1999 (hereinafter "**Kure**"). Kure describes administering a tetrahydrobiopterin compound, denoted as BH4 (manufactured by Suntory, Japan), to patients having mild hyperphenylalanemia (HPA), which is an elevation in blood phenylalanine levels (page 375). The patients were observed to be responsive to oral administration of BH4 at a frequent dosage to maintain a more constant plasma level of BH4. Page 376. Kure discovered that multiple doses of oral administration of BH4 over an extended period of time led to decreased phenylalanine levels in patients with mild phenylketonuria (PKU) (page 376). Kure further discloses that the mechanism of action of BH4 in these patients may be to increase the plasma concentration of BH4 to restore activity of the phenylalanine hydroxylase (PAH) enzyme, or to stabilize the mutant PAH molecules (page 377). Kure describes a "standard" and "modified" BH4 loading test to diagnose sensitivity of PKU patients to BH4 treatment (page 376).

Mukerji et al., U.S. Patent No. 6,428,990 (hereafter "**Mukerji**"). Mukerji describes use of a human saturase enzyme to produce new types of polyunsaturated fatty acids that can be used, e.g., to treat conditions caused by insufficient intake of polyunsaturated fatty acids. Col. 4, lines 53-57. Mukerji teaches that the polyunsaturated

fatty acids can be incorporated into nutritional compositions, a number of which are illustrated in the examples. The ingredients of such compositions will vary depending upon the intended subject with specialized needs, including subjects with metabolic conditions or disorders. Col. 11, lines 15-19. Cols. 23-38 describe a number of nutritional compositions that are useful as infant formula, milk substitutes and adult nutritional supplements that include protein, carbohydrate, oil to provide fatty acids, vitamins and minerals. Oil sources include soybean, coconut, or safflower oils (e.g., col. 23, lines 44-45, col. 27, lines 37-39, etc.). Oil sources can also include fish oils (col. 36, line 22). Carbohydrate sources include glucose, maltodextrin and other sugars, or starch (e.g., col. 11, lines 24-26, col. 27, lines 48-51, etc.). Some of the compositions are formulated with soy protein to avoid symptoms of cow milk protein allergy (e.g., col. 23, lines 25-28, col. 24, lines 39-40, etc.), or can be formulated as a gluten-free composition (e.g., col. 26, line 37 or line 66, col. 33, line 27, etc.). Compositions can be flavored, e.g. chocolate, berry, banana, cherry, strawberry, lemon or orange (e.g., col. 28, line 65 through col. 29, line 2). Mukerji discloses that nutritional formulas can be not only liquid but also may be stored as a powder that can be reconstituted with water. Col. 12, lines 6-11, col. 32, line 22. Mukerji also discloses that nutritional compositions can be in the form of liquid (e.g., cols. 23-26), snack bars (col. 26, lines 61-67), or pudding (col. 33, lines 20-27), and that nutritional compositions can be added to a variety of foods including cheese, yogurt, chocolate, candy, snacks, meats, fish, and beverages (col. 11, lines 55-57).

Nagatsu, Essays in Biochemistry, 30:15-35 (1995) (hereinafter "**Nagatsu**"). Nagatsu teaches that the concentration of tetrahydrobiopterin is a regulatory factor for tyrosine hydroxylase activity (page 25). Nagatsu also teaches that "TH [tyrosine hydroxylase] may be closely related to the pathogenesis of neurological diseases, such as dystonia and Parkinson's disease, psychiatric diseases, such as affective disorders and schizophrenia, as well as cardiovascular diseases" (page 32).

Naruse et al., U.S. Patent No. 4,778,794 (hereafter "**Naruse**"). Naruse discloses that therapeutic tetrahydrobiopterin and related compounds can be administered as tablets, capsules, powders, granules, or suspensions. Col. 3, lines 60-63.

PhenylAde Amino Acid Bars brochure (hereinafter "**Phenylade I brochure**") dated November 2002 at the bottom of the last page of the brochure. The Phenylade I brochure discloses amino acid bars that are phenylalanine-free or include a small amount of phenylalanine.

PhenylAde Amino Acid Blends brochure (hereinafter "**Phenylade II brochure**") dated May 2002 at the bottom of the last page of the brochure. The Phenylade II brochure discloses a phenylalanine-free blend of amino acids that may also include minerals and trace elements (See PhenylAde MTE Amino Acid Blend at page 2). The brochure at pages 3-4 instructs one to add the amino acid blend to a number of foodstuffs including pudding, baby food or applesauce (page 4).

Rabelink et al., US Patent Application No. 2002/0052374, published May 2002 (hereinafter "**Rabelink**"). Rabelink describes the use of at least folic acid or a folate and tetrahydrobiopterin or derivatives thereof for treating or preventing cardiovascular or neurological disorders in the modulation of nitric oxide (NO) levels. Rabelink describes that these formulations may be for oral use (paragraph 20, and Examples 1 and 6-10), and further contemplates a pharmaceutical composition comprising folate derivatives, BH4 and L-arginine for use as a modulator of NO (Abstract, paragraphs 88-91). Rabelink also describes inclusion of an antioxidant such as vitamin C in such compositions.

Schaub et al., Arch Dis Child. 53(8):674-6 (1978) (hereafter "**Schaub**"). Schaub discloses treatment of a child suffering from "atypical" phenylketonuria due to defective BH4 synthesis with tetrahydrobiopterin bishydrochloride (page 674). Administration of BH4 either intravenously or orally resulted in a decrease in serum phenylalanine levels (page 674).

Shinozaki et al., *Circ Res*. 87:566-73 (2000) (hereinafter "**Shinozaki**"). Shinozaki discloses that BH4 is a cofactor of endothelial nitric oxide synthase (eNOS) that stabilizes the dimeric, active form of the enzyme (page 566). Nitric oxide synthase (NOS) converts the semiessential amino acid l-arginine to l-citrulline and nitric oxide (NO). Shinozaki describes administration of oral BH4 (sapropterin hydrochloride) to fructose-fed, insulin-resistant rats, which suffer from a deficiency in NOS activity (page 568). BH4 decreased blood pressure and insulin levels in fed rats (page 568), and significantly raised levels of eNOS activity and decreased NO production (page 569). BH4 administration also

decreased levels of endothelial production of oxygen radicals (page 571). These results demonstrate that administration of BH4 may improve vasodilation and relieve oxidative stress (page 572). The BH4 administered was manufactured by Suntory, Japan and is sapropterin hydrochloride (page 567).

Steinfeld et al., *Eur J Pediatr.* 161:403-405 (May 2002) (hereinafter "**Steinfeld**"). Steinfeld discloses administration of BH4 to three patients having mild PKU. The patients received long term oral BH4 (approximately 12 months) and phenylanlanine levels were monitored routinely (page 403). Two of the three patients showed successful response to the long term BH4 treatment (page 403). Steinfeld describes that differential activities of PAH folding mutants contribute to differences in BH4 responsiveness, and further states that studies using BH4 substantiate the role of factors like chaperones in genetic diseases, indicating that BH4 improves PAH misfolding and acts as a chaperone molecule (page 404). Steinfeld further discloses that BH4 is used is a diagnostic tool to determine BH4 responsive PKU (page 403), and indicates that an improved test using a longer time period would be more helpful to detect individuals who response more slowly to BH4 administration (page 404).

Trefz et al., *Eur. J Pediatr.* 160:315, 2001 (hereinafter "**Trefz**"). Trefz discloses treatment of a newborn with daily doses of oral BH4 (5,6,7,8-tetrahydrobiopterin, Schircks Laboratories, Switzerland) (1st col.). Trefz reports that administration of BH4 reduced levels of phenylalanine in treated individuals. Trefz further describes a BH4 diagnostic loading test to identify BH4 responsive individuals (col. 2). Trefz further discloses that administration of BH4 may enhance the activity of mutated PAH proteins in PKU patients (col. 2).

Walter et al., *Am J Respir Crit Care Med.* 156(6):2006-10 (1997) (hereinafter "**Walter**"). Walter describes administration of BH4 to healthy human volunteers via inhalation. Walters states that BH4 is a regulatory cofactor for nitric oxide synthase (NOS) and that BH4 regulates iNOS expression by stabilization of its mRNA (page 2006). At low concentrations, BH4 was reported to restore disturbed NO-dependent vasodilation in patients with endothelial dysfunction (page 2009). The BH4 used was manufactured by Dr. B. Schircks Laboratories, Jona, Switzerland, and is (6R)-5,6,7,8-tetrahydro-L-biopterin-dihydrochloride dissolved in N-acetylcysteine 10% (page 2007, 1st col.). N-acetylcysteine

was used for galenic stabilization of BH4 tablets for long-term storage at room temperature (page 2007).

Weglage et al., *J. Inherit. Metab. Dis.* 25 321-322 (2002) (hereinafter "**Weglage**"). Weglage describes treatment of PKU patients with BH4, and indicates that in at least one patient, the initiation of a protein-restricted (low protein) diet (450 mg Phe/day) was necessary, in addition to BH4 treatment, to achieve satisfactory metabolic control (page 322).

III. Relevance of Cited Art

35 U.S.C. §102(b) states in relevant part that a patent may be granted unless the invention was described in a printed publication more than one year prior to application for patent in the United States.

35 U.S.C. §103(a) states in relevant part that a patent may not be obtained ..if the differences sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

1. Claim 1 is directed to use of at least one compound of formula I (which includes BH4), and pharmaceutically acceptable salts thereof, for production of a medication to enhance protein tolerance for the treatment of diseases due to amino acid metabolic disorders. Claim 1 is unpatentable under 35 U.S.C. §102(b) as anticipated by any one of Hennermann, Blau, Steinfeld, Kure, Trefz, Schaub, Shinozaki, Curtius, Curtius II, Curtius III or Dissing, all published more than 1 year before the filing date of the Muntau-Heger application.

1.1. Each of Hennermann (at line 5-7), Blau (at p. 18), Steinfeld (at p. 403, Kure (at p. 375), or Trefz (at col.1) report use of a tetrahydrobiopterin (BH4) compound encompassed by formula I for the treatment of phenylketonuria (PKU). PKU is defined in the Muntau-Heger application as an amino acid metabolic disorder. Patients with PKU have elevated blood levels of phenylalanine (an amino acid contained in all natural proteins) and cannot tolerate high levels of protein. Typically

such patients are treated by placing them on a low phenylalanine, low protein diet. See Erlandsen at p. 214. A number of different manufacturers market nutritional compositions specifically for patients with PKU that are either phenylalanine-free or low phenylalanine, but that contain other amino acids from protein. See, e.g., Phenylade I and II brochures.

1.2. Each of Hennermann, Blau, Steinfeld, Kure, or Trefz report that administration of BH4 to patients with PKU reduced blood levels of phenylalanine, which thus would enhance protein tolerance for these patients. Hennermann states "In summary, BH4 therapy succeeded in an increase of the daily phenylalanine tolerance of 180-1830 mg" and Hennermann reports that children with mild PKU could stop treatment with the low protein diet, indicating that BH4 administration enhances protein tolerance in therse patients.

1.3 Schaub reports that administration of tetrahydrobiopterin bishydrochloride (BH4) to a patient suffering from "atypical" phenylketonuria due to defective BH4 synthesis reduced blood levels of phenylalanine (p. 674).

1.4 Shinozaki reports administration of BH4 to insulin-resistant rats suffering from a deficiency in nitric oxide synthase (NOS) activity. Reduced NOS activity is defined in the Muntau-Heger application as an amino acid metabolic disorder. See Muntau-Heger page 2. Administration of BH4 raised levels of NOS activity and increased nitric oxide (NO) production (p 569). NOS converts the amino acid L-arginine to L-citrulline and NO.

1.5 Curtius and Curtius III report administration of BH4 to patients with depression, which is defined in the Muntau-Heger application as an amino acid metabolic disorder. Curtius also reports that others have administered BH4 to patients with hyperphenylalanemia (elevated phenylalanine levels), Parkinson's disease and dystonia (p. 658, 1st col.), all of which are defined in the Muntau-Heger application as an amino acid metabolic disorder. See Muntau-Heger page 2.

1.6 Dissing, Curtius II and Curtius III each report administration of BH4 to patients with Parkinson's disease, which is defined in the Muntau-Heger application as an amino acid metabolic disorder.

2. Claim 2 is directed to the use of claim 1 wherein the compound is selected from a group that includes the compounds 5,6,7,8-tetrahydrobiopterin and sapropterin, and particularly the hydrochloride or sulfate thereof. Claim 2 is anticipated under 35 U.S.C. §102(b) by any one of the references cited above for claim 1 which disclose administration of BH4 (5,6,7,8-tetrahydrobiopterin), and particularly Kure, Trefz, Blau, Schaub, Shinozaki, Dissing or Curtius II.

2.1. Kure disclose use of a sapropterin hydrochloride salt for treating PKU. Kure used BH4 produced by Suntory, Japan (p. 375, 3rd col.). As shown in Shinozaki (p. 567) the BH4 produced by Suntory is sapropterin hydrochloride.

2.2. Trefz, Blau and Schaub each discloses use of a 5,6,7,8-tetrahydrobiopterin hydrochloride salt for treating PKU. Trefz and Blau used BH4 produced by Schircks Laboratories, Switzerland (Trefz, 1st col.; Blau, p. 21, top). As shown in Walter (p. 2007), the BH4 produced by Schircks Laboratories is 5,6,7,8-tetrahydro-L-biopterin dihydrochloride. Schaub states that tetrahydrobiopterin bishydrochloride was used.

2.3 Shinozaki discloses use of a sapropterin hydrochloride salt to treat NOS deficiency (p. 567).

2.4. Dissing discloses use of a 5,6,7,8-tetrahydrobiopterin hydrochloride salt for treating patients with Parkinson's disease, defined as an amino acid metabolic disorder. (p. 495, 1st col.)

2.5 Curtius II discloses use of tetrahydrobiopterin dihydrochloride salt for treating patients with Parkinson's disease (p. 464).

3. Claim 3 is directed to the use of claim 1 or 2, wherein the salt is the hydrochloride or sulfate salt. Claim 3 is anticipated by the same references cited for claim 2, each of which disclose at least one form of the recited salt.

4. Claim 4 is directed to the use of claims 1-3 wherein the amino acid metabolic disorders include a variety of specific conditions, such as conditions with elevated phenylalanine or decreased tyrosine in bodily fluids, tissue or cells; conditions with reduced phenylalanine hydroxylase activity; conditions due to decreased cellular availability of catecholamines, including dystonia; neurotransmitter disorders, including schizophrenia; and phenylketonuria, particularly mild phenylketonuria and classic phenylketonuria. Claim 4 is unpatentable under 35 U.S.C. §102(b) in view of any one of Hennermann, Blau, Steinfeld, Kure, Trefz, Schaub, Curtius, Curtius II, Curtius III or Dissing.

4.1. Each of Hennermann, Blau, Steinfeld, Kure, or Trefz disclose use of BH4 to treat mild PKU. Hennermann also states that patients with classic PKU were treated with BH4 and responded (lines 10-12). PKU is considered a condition with elevated phenylalanine in bodily fluids and is also considered a condition with reduced phenylalanine hydroxylase activity, both of which conditions are recited in claim 4. (See, e.g., Trefz, 1st and 2nd col., Kure p. 375 1st col., Steinfeld p. 403, 1st col., Blau p. 18, Hennermann).

4.2. Schaub discloses use of BH4 to treat atypical PKU (age 674).

4.3. Curtius discloses that BH4 has been used to treat BH4-deficient hyperphenylalaninaemia, Parkinson's disease, and dystonia. (Curtius, page 658, 1st col.) Hyperphenylalanemia is elevated phenylalanine levels, a condition recited in claim 4. Parkinson's disease is a condition due to decreased cellular availability of catecholamines (such as reduced concentrations of dopamine in the brain) and is also a neurotransmitter disorder, both of which conditions are recited in claim 4. Dystonia is a condition recited in claim 4.

4.4. Curtius II and Curtius III both disclose use of BH4 to treat Parkinson's disease. (Curtius II, page 464; Curtius II, col. 1, lines 18-24)

4.5. Dissing also discloses use of BH4 to treat patients with Parkinson's disease. (Dissing, p. 493-494)

5. Claim 5 is directed to the use of claims 1-4 wherein the salt is a hydrochloride. Claim 5 is anticipated because the BH4 administered was a hydrochloride salt (see discussion above for claim 2).

6. Claim 6 is directed to use of a compound of formula I, which includes BH4, as a chaperone. Claim 6 is unpatentable as anticipated under 35 U.S.C. §102(b) over any one of the references cited for claim 1, including Steinfeld, Blau, Kure or Shinozaki.

6.1. The discovery of the mechanism of action of a known therapeutic compound, such as its action as a chaperone, is not patentable. MPEP 2112-2112.02 states "The discovery of a previously unappreciated property of a prior art composition, or of a scientific explanation for the prior art's functioning, does not render the old composition patentably new." The "chaperone" function of BH4 was already known. Each of Steinfeld, Blau or Kure disclose that the mechanism of action by which BH4 improves phenylalanine levels in PKU patients is by functioning as a chaperone. Shinozaki discloses that the mechanism of action by which BH4 improves NOS activity is by stabilizing the enzyme(p. 566). It is known in the art that chaperone molecules act to stabilize the molecule they act upon, and bind the molecule to induce structural changes which may increase the affinity of the molecule for a third target. See Ellis at page 259.

6.2. Steinfeld discloses that differences in responsiveness of PKU patients to BH4 treatment are likely due to "differences in cellular handling of PAH folding mutants" and that "evidence from our and previous studies substantiates the role of additional factors like **chaperones** in the phenotypic expression of genetic diseases." (p. 404; emphasis added).

6.3. Blau discloses that mechanisms underlying BH4-responsive PKU include compensation for a reduced affinity of the phenylalanine hydroxylase (PAH) enzyme for BH4, stabilization of the PAH protein, and introduction of 3D structural changes in PAH (p. 19). All of these activities are encompassed by the term "use as a chaperone." See Ellis, entire document.

6.4. Kure discloses that BH4 acts to "restore residual PAH activity and/or to stabilize the mutant PAH molecules." (p. 377, 2nd col.) This activity is encompassed by the term "use as a chaperone." See Ellis, entire document.

6.5. Shinozaki discloses that BH4 is a cofactor of endothelial nitric oxide synthase (eNOS) that stabilizes the dimeric, active form of the enzyme (p. 566). This activity is encompassed by the term "use as a chaperone." See Ellis, entire document.

7. Claim 7 is directed to the use of claim 6 wherein the compound is selected from a group that includes 5,6,7,8-tetrahydrobiopterin and sapropterin, and particularly the hydrochloride salt thereof. Claim 7 is anticipated by any one of the references cited above for claim 1 or 6, which disclose administration of BH4 (5,6,7,8-tetrahydrobiopterin), and particularly Blau, Kure or Shinozaki, which disclose use of 5,6,7,8-tetrahydrobiopterin dihydrochloride and sapropterin hydrochloride, respectively. See discussion above for claim 2.

8. Claim 8 recites the use of the chaperone of claim 6 to improve protein misfolding, particularly in the case of structural abnormalities in enzymes that require BH4 as a cofactor. Claim 8 is unpatentable as anticipated under 35 U.S.C. §102(b) over any one of Steinfeld, Blau, Kure or Shinozaki for the reasons discussed above for claim 6. Each of these references discloses that BH4 stabilizes misfolded PAH mutants or stabilizes NOS.

9. Claim 9 is directed to the use of claims 6-8 wherein the enzymes are selected from a group including phenylalanine hydroxylase (PAH), tyrosine hydroxylase, tryptophan hydroxylase, and NO synthase (NOS). Claim 9 is anticipated under 35 U.S.C. §102(b) over any one of Steinfeld, Blau, Kure, or Shinozaki. Steinfeld, Blau and Kure disclose that BH4 is a chaperone for PAH. Shinozaki discloses that BH4 is a chaperone for NOS (p. 566). See discussion for claim 6 above. Moreover, Nagatsu discloses that tetrahydrobiopterin is a regulatory factor for tyrosine hydroxylase activity (Nagatsu, page 25), and Curtius II states that BH4 is thought to play an important role in regulating the *in vivo* activities of tyrosine hydroxylase and tryptophan hydroxylase, which are rate-limiting enzymes in the synthesis of the catecholamines and serotonin, respectively (Curtius II, page 463).

10. Claim 10 is directed to the use of claims 6-9 wherein the chaperone is used as a neurotransmitter and/or messenger enhancer, for instance in conditions with decreased serotonin or dopamine in bodily fluids and conditions with decreased phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, or nitric oxide (NO) synthase activity. Claim 10 is unpatentable as anticipated under 35 U.S.C. §102(b) by any one of Dissing, Walter or Blau.

10.1. Dissing describes administration of BH4 as a neurotransmitter to patients suffering from Parkinson's disease (p. 494, 2nd col.). Dissing further describes that Parkinson's patients have decreased levels of the catecholamines dopamine and serotonin in bodily fluids (p. 494, 1st col.).

10.2. Walter describes administration of BH4 to conditions with decreased nitric oxide synthase (NOS) activity, a deficiency which is associated with endothelial cell dysfunction (p. 2006, 1st col.). Walter further describes that BH4 stabilizes NOS messenger RNA, i.e. is a messenger enhancer (p. 2006, 1st col.).

10.3 Blau describes that the mechanisms underlying BH4 responsiveness in phenylalanine hydroxylase (PAH) deficiency, including PKU, include induction of PAH gene expression (p. 19), i.e. acting as a messenger enhancer.

11. Claim 11 is directed to use of a compound of formula I, or a pharmaceutical salt thereof, as a neurotransmitter or a messenger enhancer, particularly for catecholamines and/or serotonin and/or dopamine and/or nitric oxide. Claim 11 is anticipated under 102(b) by any one of Dissing, Walter or Blau for the same reasons described above for claim 10.

12. Claim 12 is directed to the use of claim 11 wherein the compound is selected from a group that includes 5,6,7,8-tetrahydrobiopterin and sapropterin, and particularly the hydrochloride thereof. Claim 12 is anticipated by Dissing, which discloses use of BH4 (5,6,7,8-tetrahydrobiopterin), or Walter or Blau, which each disclose use of 5,6,7,8-tetrahydrobiopterin dihydrochloride, for the reasons described above for claim 11.

13. Claim 13 is directed to a composition containing a compound of formula I (which includes BH4) or a pharmaceutically acceptable salt thereof, and also comprising at

least one amino acid selected from the group of essential amino acids as well as non-essential amino acids, particularly arginine, cysteine, especially acetylcysteine, and tyrosine. Claim 13 is unpatentable under 35 U.S.C. §102(b) as anticipated by any one of Rabelink, Walter or Dissing.

13.1. Rabelink discloses formulation of a composition comprising BH4 or derivatives thereof, and the amino acid L-arginine. Rabelink (paragraphs 88-91). Walter discloses a composition comprising BH4 and the amino acid N-acetylcysteine. (Walter, p. 2007) Dissing discloses administration of BH4 in conjunction with the amino acid tyrosine to patients having Parkinson's disease (Dissing, p. 495).

14. Claim 14 is directed to a composition of claim 13 that contains (a) an essential amino acid selected from the group consisting of: isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine, and histidine and (b) at least one of the amino acids alanine, arginine, asparaginic acid, asparagine, cysteine, especially acetylcysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine. Claim 14 is obvious under 35 U.S.C. §103 over Curtius, which reports that a patient suffering from depression was concurrently treated with BH4 and with the amino acids tryptophan (an essential amino acid from the first list) and tyrosine (an amino acid from the second list) (Curtius, page 658, 1st col.). Combining the concurrently administered cofactor and amino acid supplements in a single composition would have been obvious to a worker of ordinary skill in the art based on the disclosure of Curtius and the level of knowledge in the art.

14.1 Claim 14 is also obvious for the same reasons described below with respect to claim 19, i.e. motivation to combine BH4 or sapropterin with nutritional compositions, such as PhenylAde Amino Acid Blend, other nutritional compositions, or ordinary food. The Amino Acid Blend contains the amino acids recited in claim 14. PhenylAde II brochure, page 2. Hansen also discloses nutritional compositions that contain protein or the amino acids listed in claim 14. Hansen, col. 4, line 66 - col. 5, line 1. A worker of ordinary skill would have been motivated to combine the disclosure of Hansen with the disclosure in PhenylAde I and II with a reasonable expectation of success at making a nutritional composition having BH4, in order to treat a patient having PKU and facilitate compliance with a low-phenylalanine diet.

15. Claim 15 is directed to a composition of claim 13 or 14 that contains additional carbohydrates and/or vitamins. Claim 15 is anticipated under 35 U.S.C. §102(b) by Rabelink. Rabelink discloses a composition comprising BH4, the amino acid arginine and folate (paragraphs 88-91). It is well known in the art that folate is a member of the vitamin group of molecules (Hansen, col. 5, lines 22-29).

15.1 Claim 15 is also obvious for the same reasons described below with respect to claim 19, i.e. motivation to combine BH4 or sapropterin with other nutritional compositions. Hansen discloses that nutritional compositions, with which medication may be combined, can include vitamins and minerals. Hansen, col. 5, lines 22-29.

16. Claim 16 is directed to a composition of claim 13-15 formulated to be administered orally or intravenously. Claim 16 is anticipated under 35 U.S.C. §102(b) by Rabelink. Rabelink discloses preparation of a pharmaceutical composition for oral use comprising BH4, the amino acid arginine and, optionally folate (paragraphs 88-91).

16.1 Claim 16 is also obvious for the same reasons described below with respect to claim 19, i.e. motivation to combine BH4 or sapropterin with nutritional compositions, such as PhenylAde Amino Acid Blend, other nutritional compositions, or ordinary food in order to make a formulation palatable to treat patients with PKU and/or facilitate compliance with a low-phenylalanine diet. PhenylAde Amino Acid Blend is taken orally, as is ordinary food. See PhenylAde II brochure. Hansen discloses that medication can be combined with nutritional compositions that are consumed by the subject. Hansen, col. 3, line 51.

17. Claim 17 is directed to a composition of claim 16 wherein the formulation is a powder, tablet, capsule, coated tablet, drops or for topical use, as well as a solution for intravenous administration. Claim 17 is anticipated under 35 U.S.C. §102(b) by Rabelink. Rabelink discloses the formulation of a composition for oral administration comprising BH4, the amino acid arginine and, optionally folate. Rabelink describes that an oral formulation may be a powder, tablet, capsule, drops (paragraph 20) and also indicates that the composition may be applied topically or modified for parenteral administration, which includes intravenous administration (paragraphs 21-22, and Examples 3-5).

17.1 Claim 17 is also obvious in view of Naruse, which discloses that therapeutic tetrahydrobiopterin and related compounds can be administered as tablets, capsules, powders, granules, or suspensions. Col. 3, lines 60-63. A worker of ordinary skill reading the disclosure of Rabelink, in view of Naruse, would be motivated to formulate a composition having BH4 in a tablet, capsule, or powder in order to make administration of BH4 to patients with PKU easier and to facilitate better compliance with the low-phenylalanine diet.

18. Claim 18 is directed to a composition of claim 14-17 with a galenic pharmaceutical adjuvant. Claim 18 is anticipated under 35 U.S.C. §102(b) or obvious under 35 U.S.C. §103(a) in view of the disclosure in Walters that N-acetylcysteine was used for galenic stabilization of BH4 tablets. Acetylcysteine is set out in claim 14 as a preferred amino acid in the composition. Walters discloses that acetylcysteine is a stabilizer in BH4 tablets. Thus, a worker of skill in the art would readily combine the teachings of Walter with the knowledge in the art to make a BH4 formulation containing acetylcysteine for galenic stabilization, in order to make the medication more stable and effective when administered to patients with PKU.

19. Claim 19 is directed to a composition of claims 13-18 that is a dietary composition with adjuvants usual in foodstuffs technology. Claim 19 is anticipated under 35 U.S.C. §102(b) in view of the disclosure in Curtius III that the BH4 was mixed with orange juice, a foodstuff. Alternatively, claim 19 is obvious under 35 U.S.C. §103(a) in view of Bates, Isaacs, Hansen or the PhenylAde II brochure which teach that medications that are administered orally can be combined with food or formulated with nutritional compositions.

19.1. Bates and Isaacs disclose that medications can be administered by mixing them in formula, beverages or foods, providing motivation to combine medications and food. The references indicate that medications can be combined with a number of foods including fruit juice, ice cream or soft food (Bates, page 6) or rice formula, cereal, pudding, applesauce, mashed potatoes, yogurt, or jello (Isaacs, page 38, 3rd col.). Thus, a worker of ordinary skill in the art reading Bates or Isaacs, would have been motivated to prepare a dietary composition containing a medication, such as BH4 and food, including adjuvants usual in foodstuffs technology, particularly

since Curtius III discloses that BH4 was mixed with a foodstuff, in order to make the formulation more palatable to patients requiring low-phenylalaine food stuffs which in turn would facilitate compliance with a low-phenylalanine diet.

19.2. Hansen discloses that a preferred means of administering myo-inositol, the medication that is the subject of the Hansen patent, in order to lower glucose levels in diabetic patients who may be on a carbohydrate limiting diet is by incorporating it into an infant or adult nutritional composition. Hansen, col. 3, lines 48-51. Thus it would have been obvious to a worker of ordinary skill in the art reading Hansen in view of the knowledge in the art that another orally administered medication, such as BH4, could be incorporated into an infant or adult nutritional composition for administration to an individual having a metabolic disorder in order to facilitate compliance to a phenylalanine limiting diet.

Moreover such a combination of two orally administered compositions is naturally motivated by a desire to reduce the number of separate cmpsitions ingested by a patient.

19.3. Patients suffering from phenylketonuria frequently eat special phenylalanine-free or low phenylalanine medical foods such as PhenylAde Amino Acid Blend. See PhenylAde MTE Amino Acid Blend at page 3 of the Phenylade II brochure. It would have been obvious that an orally administered medication, such as BH4, could be incorporated into this orally administered special nutritional composition. Following the instructions in the PhenylAde II brochure, it would also have been obvious to combine BH4, the PhenylAde Amino Acid Blend, and other conventional foodstuffs. Page 4 of the Phenylade II brochure instructs one to add the amino acid blend to a number of foodstuffs including pudding, baby food or applesauce. Bates and Isaacs teach that medications may be added to soft foods, such as pudding, baby food or applesauce. Thus, it would have been obvious to a worker of ordinary skill reading Hansen, which discloses incorporating a medication into an infant or adult nutritional composition, further in view of the disclosure of PhenylAde II, to prepare a food composition containing PhenylAde MTE Amino Acid Blend and a medication such as BH4 for a patient that is being treated with both BH4 and low-

phenylalanine diet. See, e.g., Weglage at page 322, which states that in some cases a combination of BH4 treatment and phenylalanine-restricted, or low phenylalanine, diet was necessary to achieve control of phenylalanine levels

20. Claim 20 is directed to a composition of claims 13-19 that contains additional minerals and/or electrolytes. Claim 20 is obvious for the reasons discussed above with respect to claim 19, and additionally because supplementation of food products with minerals and/or electrolytes is well known. For example, PhenylAde MTE Amino Acid Blend (see PhenylAde II brochure) contains added minerals and trace elements. Hansen also discloses that the nutritional compositions containing medication may contain added minerals and vitamins. Hansen, col. 5, lines 22-29.

21. Claim 21 is directed to a composition of claim 13-20 that contains additional phenylalanine and is obvious for the same reasons described above for claims 13-20, in view of the Phenylade I brochure. This brochure shows an amino acid bar (Chocolate Crispy) that has some phenylalanine.

22. Claim 22 is directed to a composition of claim 13-21 that contains additional L-carnitine. Claim 22 is obvious for the same reasons described above for claims 13-21. Phenylade Amino Acid blend contains L-carnitine. See PhenylAde II brochure.

23. Claim 23 is directed to a composition of claim 13-22 that contains myoinositol and choline. Claim 22 is obvious because Hansen teaches that nutritional compositions (with may incorporate medication) include myo-inositol (col. 1, lines 33-40) and choline (col. 5, line 26).

24. Claim 24 is directed to a composition of claim 13-19 which contains antioxidants, particularly Vitamin C. Claim 24 is anticipated under 35 U.S.C. §102(b) by Rabelink, which discloses a composition comprising BH4, the amino acid arginine, and optional ingredients including a B vitamin folate and an antioxidant such as ascorbic acid, i.e. vitamin C (Rabelink, paragraph 74).

25. Claim 25 is directed to the composition of claim 13-24 wherein the compound is selected from a group that includes 5,6,7,8-tetrahydrobiopterin, particularly the

hydrochloride thereof. Claim 25 is anticipated or obvious for the reasons described above with respect to claim 13-24. Claim 25 is anticipated by Rabelink, Walters or Dissing, which disclose compositions containing BH4 (5,6,7,8-tetrahydrobiopterin) and an amino acid. Walters specifically states that the BH4 used was a 5,6,7,8-tetrahydrobiopterin dihydrochloride. Rabelink also discloses optional inclusion of a vitamin and antioxidant.

26. Claim 26 is directed to the use of a compound of formula I as a dietary supplement and is obvious for the same reasons discussed above for claim 19.

27. Claim 27 is directed to the use of claim 26 wherein the compound is selected from a group that includes the compounds 5,6,7,8-tetrahydrobiopterin and sapropterin, and particularly the hydrochloride thereof and is obvious for the same reasons discussed above with respect to claim 19, in view of the references discussed with respect to claim 2 (showing administration of hydrochloride salts of 5,6,7,8-tetrahydrobiopterin and sapropterin).

28. Claim 28 is directed to a special food based on mixtures of essentially phenylalanine-free mixtures, that contains at least one compound of formula I. Claim 28 is obvious for the same reasons discussed above for claim 19.

29. Claim 29 is directed to the special food of claim 28 wherein the compound is selected from a group that includes the compounds 5,6,7,8-tetrahydrobiopterin and sapropterin, and particularly the hydrochloride thereof. Claim 29 is obvious for the same reasons discussed with respect to claim 19, in view of the references discussed with respect to claim 2 (showing administration of hydrochloride salts of 5,6,7,8-tetrahydrobiopterin and sapropterin).

30. Claim 30 is directed to the special food of claim 28 or 29 that contains additional carbohydrates, (particularly glucose, maltodextrin, starches) and/or fats, (such as fish oil, particularly salmon oil, herring oil, mackerel oil, or tuna fish oil). Claim 30 is obvious because Hansen discloses that nutritional compositions (which may incorporate medication) include a carbohydrate source and a lipid source. Hansen, col. 3, lines 64-66. Exemplary carbohydrates include glucose, maltodextrin and starches such as rice, corn and tapioca starches. Hansen, col. 4, lines 35-39. Exemplary fats include fish oil. Hansen, col. 4, line 51. Mukerij also discloses similar components for nutritional compositions, in which

carbohydrate sources include glucose, maltodextrin and other sugars, or starch (e.g., Mukerji, col. 11, lines 24-26, col. 27, lines 48-51, etc.) and exemplary fats include fish oil (e.g., Mukerji, col. 36, line 22). Thus, it would have been obvious to a worker of ordinary skill in the art reading Hansen, that another orally administered medication, such as BH4, could be incorporated into a nutritional composition described above, e.g., with an additional lipid and a carbohydrate source, in order to make the formulation more palatable to patients requiring low-phenylalanine food stuffs.

31. Claim 31 is directed to the special food of one of claims 28-30 that is hypoallergenic and/or essentially gluten-free. Claim 31 is obvious because Mukerji discloses that nutritional compositions can be formulated with soy protein to avoid symptoms of cow milk protein allergy (e.g., Mukerji, col. 23, lines 25-28, col. 24, lines 39-40, etc.), or can be formulated as a gluten-free composition (e.g., Mukerji, col. 26, line 37 or line 66, col. 33, line 27, etc.). A worker of ordinary skill would have been motivated to substitute any known nutritional composition for any exemplary nutritional compositions disclosed in Hansen to treat a patient having a metabolic disorder. Mukerji teaches, for example, hypoallergenic/gluten-free compositions. Thus, the combination of Hansen and Mukerji teaches the combination of oral medications such as BH4 with hypoallergenic or gluten-free nutritional compositions such as disclosed in Mukerji, to accommodate a patient with PKU and additional food allergies.

32. Claim 32 is directed to the special food of one of claims 28-31 that is an infant formula. Claim 32 is obvious for the same reasons discussed with respect to claims 30 and 31, because Hansen discloses that nutritional compositions (which may incorporate medication) include infant formula. Hansen, col. 3, line 61.

33. Claim 33 is directed to the special food that is a powder, particularly a freeze-dried powder. Claim 33 is obvious for the same reasons discussed with respect to claims 30 and 31, because Mukerji discloses that nutritional formulas can be not only liquid but also may be stored as a powder that can be reconstituted with water. Mukerji, col. 12, lines 6-11, col. 32, line 22.

34. Claim 34 is directed to the special food that contains additional fatty acid supplements, particularly unsaturated fatty acids, preferably omega 3 fatty acids, especially

alpha-linolenic acid, docosahexaenoic acid, eicosapentaenoic acid, or omega 6 fatty acids, in particular arachidonic acid, linoleic acid, or linolenic acid; or oleic acid. Claim 34 is obvious for the same reasons discussed with respect to claims 30 and 31, because Hansen discloses that nutritional compositions (which may incorporate medication) can include these named fatty acids of the omega 3 and omega 6 categories (arachidonic acid, linoleic acid, palmitic acid, stearic acid, docosahexaenoic acid, eicosapentaenoic acid, linolenic acid, oleic acid). Col. 4, lines 54-56. Thus, it would have been obvious to a worker of ordinary skill in the art reading Hansen, that another orally administered medication, such as BH4, could be incorporated into a nutritional composition as described above, in order to make a formulation more palatable to patients requiring low-phenylalanine food stuffs.

35. Claim 35 is directed to the special food that contains fish oil additives, particularly salmon, herring, mackerel or tuna fish oil. Claim 35 is obvious because Hansen discloses that nutritional compositions (which may incorporate medication) include fish oil. Col. 4, line 51. Mukerji also discloses that oil sources for nutritional compositions can include fish oil additives (col. 36, line 22). Thus, it would have been obvious to a worker of ordinary skill in the art reading Hansen, that another orally administered medication, such as BH4, could be incorporated into a nutritional composition having fish oil as described in Hansen, in order to make a formulation more palatable to patients requiring low-phenylalanine food stuffs.

36. Claim 36 is directed to the special food of claims 28-35 that can be used as a milk substitute, particularly for nursing infants. Claim 36 is obvious because Hansen discloses that nutritional compositions (which may incorporate medication) include infant formula, and that such compositions can be milk-based or soy-based (as a milk substitute). Hansen, col. 3, lines 61-63. Mukerji also discloses nutritional compositions that are used as a milk substitute, particularly for nursing infants. Mukerji, cols. 23-26. Thus, it would have been obvious to a worker of ordinary skill in the art reading Hansen, that another orally administered medication, such as BH4, could be incorporated into a nutritional composition used as a milk substitute as described in Hansen, in order to make a formulation more palatable to patients requiring low-phenylalanine food stuffs.

37. Claim 37 is directed to the special food of claim 36 wherein the milk substitute has a fat content of 90% triglycerides and 10% mono- and di-glycerides. To the extent that nutritional compositions known in the art have such a fat content, this claim is obvious for reasons discussed above for claims 28-35.

38. Claim 38 is directed to the special food of claim 37 wherein the fat component includes plants oils, particularly safflower oils and/or soybean oil and/or coco oil. Claim 38 is obvious because Hansen discloses that nutritional compositions (which may incorporate medication) include plant oils, including safflower, soybean and coconut oils. Hansen, col. 4, lines 49-51. Mukerji also discloses that oil sources for nutritional compositions include soybean, coconut, or safflower oils (e.g., Mukerji, col. 23, lines 44-45, col. 27, lines 37-39, etc.). Thus, it would have been obvious to a worker of ordinary skill in the art reading Hansen, that another orally administered medication, such as BH4, could be incorporated into a nutritional composition described above in order to make the formulation more palatable to patients requiring low-phenylalanine food stuffs.

39. Claim 39 is directed to the special food of claims 28-38 that is formed as a milk drink mix, particularly a fruit-flavored or chocolate drink mix. Claim 39 is obvious because Hansen discloses that nutritional compositions, with which medications may be combined, may be a variety of flavors including chocolate, banana or strawberry. Hansen, col. 5, lines 46-47. Mukerji also discloses that nutritional compositions can be flavored, e.g. chocolate, berry, banana, cherry, strawberry, lemon or orange (e.g., Mukerji, col. 28, line 65 through col. 29, line 2). Thus, it would have been obvious to a worker of ordinary skill in the art reading Hansen, that another orally administered medication, such as BH4, could be incorporated into a nutritional composition comprising a flavored mix as described in Hansen, in order to make a formulation more palatable to patients requiring low-phenylalanine food stuffs.

40. Claim 40 is directed to a special low-phenylalanine foodstuff containing a low-protein basic food as well as at least one compound of formula I. Claim 40 is obvious for the same reasons described above for claim 19. The PhenylAde I and II brochures disclose special low-phenylalanine foodstuffs. Hansen also discloses that nutritional compositions may be low protein, e.g. ranging from 7-30% protein, for example, 10-15% or

12% protein. Hansen, col. 4, lines 9-21. Thus, it would have been obvious to a worker of ordinary skill in the art reading Hansen, in view of PhenylAde II, that BH4 could be incorporated into a low-protein nutritional composition as described in Hansen, in order to make a formulation more palatable to patients requiring low-phenylalanine food stuffs or to facilitate compliance with a low-protein diet in these patients.

41. Claim 41 is directed to the special food of claim 40 wherein the compound is selected from a group that includes the compounds 5,6,7,8-tetrahydrobiopterin and sapropterin, and particularly the hydrochloride thereof. Claim 41 is obvious for the same reasons as for claims 19 and 40 in view of the references discussed with respect to claim 2 (showing administration of hydrochloride salts of 5,6,7,8-tetrahydrobiopterin and sapropterin).

42. Claim 42 is directed to a special low-phenylalanine foodstuff according to Claim 40 or 41 that is selected from the group of convenience foods; pasta, particularly noodles; baked goods, particularly bread, cakes, and cookies; sweets, particularly chocolate, hard candies, and ice creams; and drinks, particularly milk substitutes in the form of drink mixes, particularly fruit-flavored or chocolate drink mixes; and beer. Claim 42 is obvious for the same reasons described above for claims 19, 40 and 41 above in view of the disclosure that low-phenylalanine foodstuffs can be beverages, pudding, or bars (PhenylAde I or II brochures), and the disclosure of Hansen that nutritional compositions can be fruit- or chocolate-flavored (Hansen, col. 5, lines 46-47). The particular choice of food form is not inventive. Mukerji also discloses that nutritional compositions can be in the form of liquid (e.g., cols. 23-26), snack bars (col. 26, lines 61-67), or pudding (col. 33, lines 20-27), and that nutritional compositions can be added to a variety of foods including cheese, yogurt, chocolate, candy, snacks, meats, fish, and beverages (col. 11, lines 55-57).

43. Claim 43 is directed to a diagnostic tool for the diagnosis of sensitivity to BH4 in amino acid metabolic diseases, containing at least one compound of formula I (a formula which includes BH4). Claim 43 is unpatentable under 35 U.S.C. §102(b) as anticipated by any one of Steinfeld, Kure, Trefz or Blau.

43.1. Steinfeld discloses the use of BH4 as a diagnostic tool, in a BH4 loading test, to determine if a patient with PKU is responsive (i.e., sensitive) to BH4

(p. 403, 1st col.). Kure describes use of BH4 as a diagnostic tool, in a standard and modified form of a BH4 loading test, to determine if a patient with PKU is responsive to BH4 (pp. 376-377). Trefz also discloses a BH4 loading test (2nd col.). Blau also discloses a BH4 loading test to discriminate BH4-responsive patients from BH4-nonresponsive patients (p. 18).

44. Claim 44 is directed to the use of claim 10 (chaperone or neurotransmitter or messenger enhancer) wherein the conditions include specific conditions described in Dissing, Walter or Blau, such as phenylketonuria, particularly mild PKU and classic PKU, conditions caused by decreased cellular availability of catecholamines, neurotransmitter disorders, conditions caused by reduced cellular availability of dopamine or serotonin, particularly Parkinson's disease, or conditions with reduced NO synthase activity, particularly endothelial dysfunctions. Claim 44 is thus anticipated by Dissing, Walter or Blau for the same reasons described above for claim 10.

IV. Conclusion

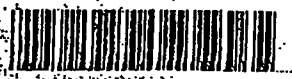
The Examiner's attention is respectfully drawn to the contents of the publications cited herein, all of which were published more than one year before the filing date of the Muntau-Heger application and thus are prior art under 35 U.S.C. §102(b). For the reasons described herein, all claims of PCT/EP2003/014262 and its corresponding U.S. national phase application, U.S.S.N. 10/539,842, are unpatentable under 37 CFR § 102(b)/103.

Dated: July 20, 2005

Respectfully submitted,

By Li-Hsien Rin-Laures

Li-Hsien Rin-Laures, M.D.
Registration No.: 33,547
MARSHALL, GERSTEIN & BORUN LLP
233 S. Wacker Drive, Suite 6300
Sears Tower
Chicago, Illinois 60606-6357
(312) 474-6300



Children's Medications

A Parent's Guide

RICHARD D. BATES, PHARM.D.

Assistant Professor
Department of Pediatric Pharmacy
College of Pharmacy
Wayne State University
Children's Hospital
Detroit, Michigan

and

MARCIA NASH, PHARM.D.

Professor, Pharmacy and Pediatrics
College of Pharmacy
The Ohio State University
and Children's Hospital
Columbus, Ohio

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MEDICATION STORAGE

What is the ideal place for storing medicine? Several factors are important to consider.

First, and most important, **keep *all* medicine out of the reach of children.** Children are extremely curious, not only as infants, but also when they get older. The curiosity of children should never be underestimated, whatever their age. Poison Control Centers frequently receive calls regarding children of all ages who, for no apparent reason, have swallowed a potentially lethal substance.

All medicines should be kept in a place that is very inaccessible to children, whether this means placing them on the top shelf of a child-proof cabinet or in a locked safe. Do not expect a child-proof container to prevent the child from getting the medicine. A child-proof container is one that only takes longer for a young child to open than an adult.

If the medicine is kept in a locked cabinet that is accessible to children, be sure the cabinet is always locked, whether or not you are in the room. If the cabinet is not locked when you are in the room, it is no better than placing the medicine on the floor in front of the child. It may take less than a minute for a child to get the medicine and take enough to cause serious harm.

What about medicine that needs to be refrigerated? It is impractical to lock a refrigerator. However, some basic steps can be taken to decrease the chance of the child getting into the medicine. The easiest is to put the medicine on the top shelf of the refrigerator. If the child can reach or get to the top shelf, the medicine can be hidden behind something on that shelf. The medicine then will be out of the child's sight and, hopefully, out of mind. If you believe it is necessary to make it more difficult for the child to open the refrigerator, velcro straps that can be attached to the door are available. However, this provides only limited protection.

Second, moisture and direct sunlight may damage some medicines, making them less effective. Unless otherwise directed, medicine always should be kept in a cool, dry place away from sunlight.

Third, always keep the medicine in the original container it was dispensed in from the pharmacy. It is required by law that all medicine dispensed from the pharmacy be placed in child-proof containers unless the patient specifies otherwise. As mentioned earlier, a child-proof container is not a guarantee that a child cannot open it. It only means that it usually takes young children longer to open it than an adult. It is this extra time that may allow you to stop the child from getting hurt.

Even with all the care you take in storing medicines, you may still find young children with an open bottle. If this occurs, you *must* do the following:

1) **Remain calm.** If you panic, that does not help the child and may even delay the administration of the proper treatment that may save the child's life.

2) **Take the medicine container and medicine away from the child, but keep the container near you.**

3) **Do not let the child leave your sight.** Some medicine may cause the child to become dizzy and fall or pass out. It is best that he remains near you, sitting or lying on the floor. If he refuses, do not waste time trying to get him to sit or lie on the floor, but at least keep him in your sight.

4) **Call 911 or the Poison Control Center before you do anything else.** Never try to make the child vomit unless otherwise directed to do so by a specially trained health professional. Keep the Poison Control Center's number readily available.

5) **When talking to 911 or the Poison Control Center, remain calm and give them all the information requested.** Some of the questions they ask may seem unnecessary to you, but it is necessary to determine if the child has been poisoned or not.

Emergency Numbers

Poison Control Center
Telephone: _____

Hospital Emergency Room
Telephone: _____

Hospital Emergency Room
Telephone: _____

Physician
Telephone: _____

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ontrol Center
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MEDICATION ADMINISTRATION: THE CORRECT WAY

Today, medicine comes in a variety of dosage forms. A dosage form is the form the medicine comes in, such as capsule, tablet, liquid. Listed below are a variety of different dosage forms. Under each one, detailed instructions are provided on how to administer it correctly. If at any time you have difficulty understanding the directions for administering the medicine call the child's physician or a pharmacist for help.

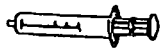
ORAL

Liquids

Liquid medications are probably the most convenient and easy way to give medicine to infants and young children. When giving a liquid medicine, never use an ordinary kitchen teaspoon or tablespoon. Kitchen utensils usually do not deliver the correct volume of liquid; therefore, underdosage or overdosage may occur. Always use a dropper, spoon, syringe, or measuring cup manufactured specifically for giving medicine. This is the only way to be sure that the correct dose is given. These usually are marked so as little as one-fourth, or less, of a teaspoon of liquid can be given accurately.

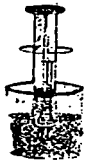
One problem when using these devices is how to get the medicine into them without making a mess. It may be difficult, unless you have been shown the proper way. The following steps should make it easier for you to administer liquid medicine.

Oral Medication Syringe

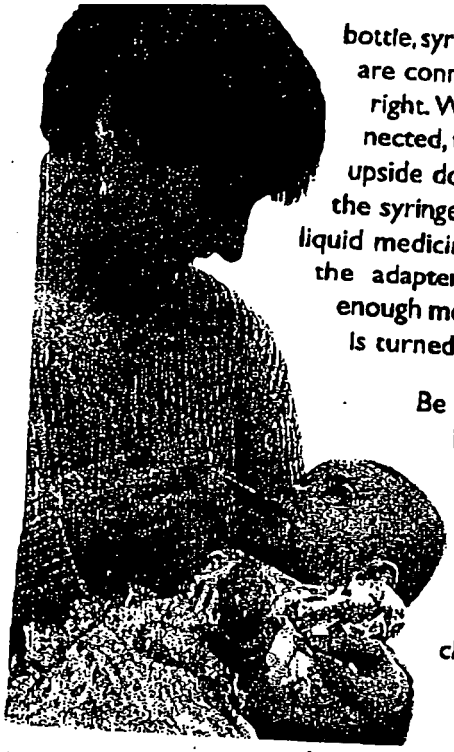


There are basically two proper ways to put the correct amount of medicine into the syringe.

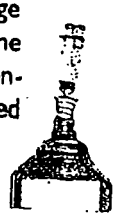
1) The easiest is to pour a small volume of the liquid medicine into a medicine cup, or any other small cup you have. Put the tip of the syringe into the liquid in the cup and pull back on the plunger to withdraw the proper volume of liquid. Make sure that the tip of the syringe remains under the top level of the liquid so air is not drawn into the syringe. The remaining liquid should then be poured back into the medicine bottle.



2) Another method, which may take some practice, is to use a syringe adapter. This is a triangular shaped plastic device. The medicine



bottle, syringe adapter, and syringe are connected as shown to the right. When all three are connected, the entire unit is turned upside down. Pulling back on the syringe plunger allows the liquid medicine to flow through the adapter and into the syringe. When enough medicine is in the syringe, the unit is turned over and taken apart.



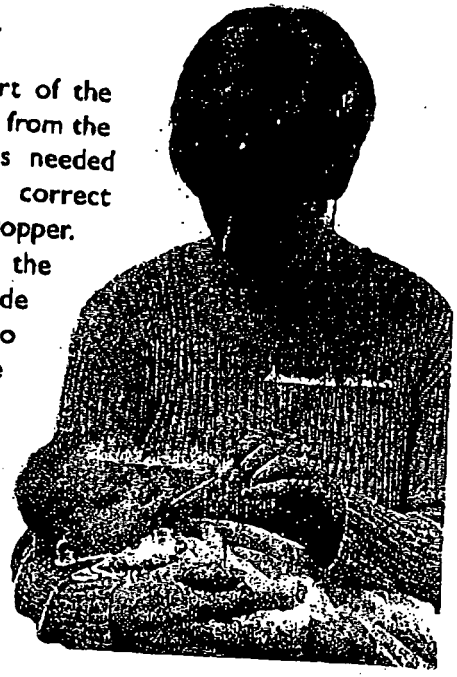
Be sure to squirt the liquid medicine into the side of the child's mouth. Squirting it directly into the back of the throat may cause gagging and coughing. Squirt the liquid slowly enough to allow the child to swallow naturally.

The syringe should be rinsed with warm water after each use.

Oral Medicine Dropper

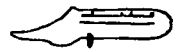
The dropper is usually part of the medicine bottle you receive from the pharmacy. When a dose is needed simply withdraw the correct volume into the dropper. Be sure to drop the liquid into the side of the mouth to avoid possible gagging and coughing.

The dropper should be rinsed with warm water if it is not a part of the medicine bottle.



Medicine Spoon

The easiest way to get liquid medicine into a medicine spoon is to hold the spoon upright. With the spoon upright, and using the markings on the side as guides, pour the correct volume of medicine into the spoon. Before giving the medicine, you may want to tell the child to pretend she is drinking a glass of milk or juice. Put the spoon up to her lips and tilt it. The liquid should flow into the child's mouth slowly enough to allow normal swallowing.



The spoon should be rinsed with warm water after each use.

One problem that occasionally arises when trying to administer liquid medicine to a child is that it tastes bad. Some medicines taste relatively good, like amoxicillin; others taste bad, like prednisolone. When a child finds that a liquid medicine tastes bad, she may not want to take any liquid medicine, especially any that look like the original one.

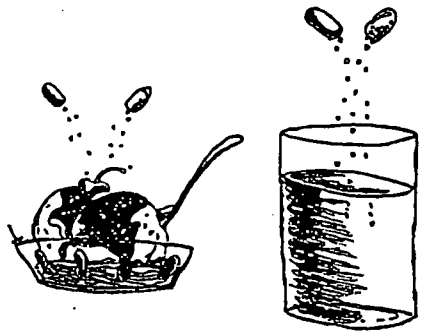
A way to overcome this is by mixing the medicine in a small volume of water or fruit juice. This will help mask the taste of the medicine so the child can take it. If this is done, the child must drink the entire mixture to be sure the whole dose was taken. However, not all liquid medicine should be diluted in water or fruit juice. Contact the child's physician or a pharmacist for guidance.

If the child spits the medicine out or allows it to "drool" out of his mouth, do not readminister it. Mark it as a partial dose; the physician should then be notified to determine further action.

Tablets and Capsules

Tablets and capsules are usually the easiest and cleanest way to give medicine to older children. As a rule, unless otherwise instructed by a physician or pharmacist, they should always be swallowed whole with water. This is because some tablets and capsules have protective properties that may either protect the stomach from becoming upset or the medicine from being harmed by the acid in the stomach. Others may have long-acting properties that allow the medicine to be given one or two times a day. If these tablets and capsules are chewed, cut, crushed, or opened, the protective and/or long-acting properties may be destroyed and the drug then could possibly harm the child. To be sure the correct amount of medicine is being given in the safest way, tablets and capsules should **not** be crushed, chewed, cut, or opened unless otherwise instructed by a physician or pharmacist.

When a certain medicine is not manufactured in a liquid form, a tablet or capsule is prescribed. This is a problem for infants or young children, since they are unable to swallow a tablet or capsule. Many tablets or capsules can be changed into liquid medicine by the pharmacist at the pharmacy. Others do not have to be converted to liquid medicine, because the tablet or capsule can be mixed with liquid or food at home. To do this, just crush the tablet or open the capsule and sprinkle the powder into a small volume of water or fruit juice or over a small amount of ice cream or soft food. To be sure the entire dose is taken, the child must drink or eat the entire mixture. Mixing medicine with food or liquid should be done only under the direction of a physician or pharmacist.



OTIC (Ear)

A. Wash your hands before giving the medicine.

B. Warm the medicine to **body temperature** by holding the bottle between your hands for several minutes.

C. Place the child on his side so the affected ear is easily accessible.

D. If ear drainage is present, gently clean the ear canal with a cotton swab.

E. Straighten the ear canal by the following methods:

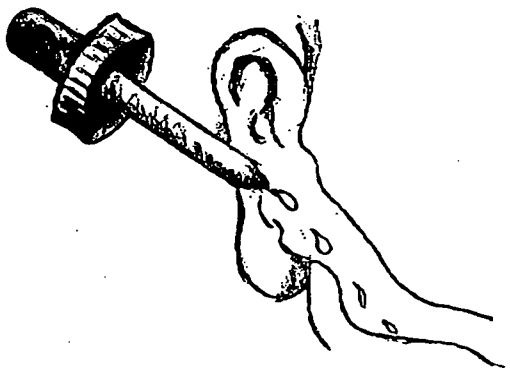
Child younger than 3 years: hold the ear lobe and pull down and back.

Child older than 3 years: hold the upper part of the ear and pull up and back.

F. Without touching the dropper to the ear, drop the prescribed number of drops into the ear. It is best if the drops are placed onto the side of the ear canal and not dropped directly down the ear canal.

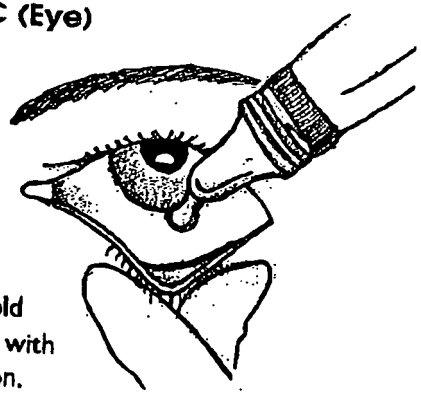
G. Have the child remain on his side for about 5 minutes.

H. If the other ear requires drops, repeat steps C to G.



OPHTHALMIC (Eye)

Drops



A. Wash your hands before giving the medicine.

B. Warm the medicine to **room temperature.**

C. Clean all secretions and old medication from the eyes with moistened gauze or cotton.

D. Place the child in a sitting or standing position with her head tilted back.

E. Be careful not to touch the dropper to the eye or eyelashes.

F. While the child is looking toward the ceiling, use the index finger and thumb to gently pinch and pull down the lower eyelid to create a "pouch." Drop the prescribed number of drops of the medicine into the pouch and not directly onto the eye.

G. Have the child close her eyes for 1 to 2 minutes.

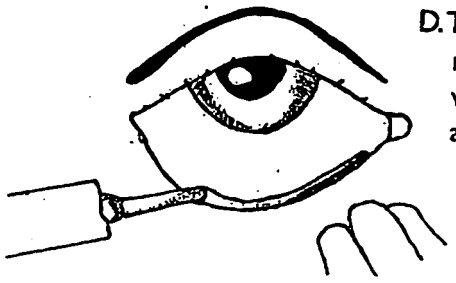
H. If other eye drops also are prescribed, wait at least 5 minutes before giving the second medicine.

Ointment

A. Wash your hands before giving the medicine.

B. Warm the medicine to **room temperature.**

C. Clean all secretions and old medication from the eyes with moistened gauze or cotton.



D. Tell the child that the ointment may cause blurred vision, but this is normal and will go away quickly.

E. Place the child on his back with his head tilted back.

F. Be sure not to touch the tip of the tube to the eye or eyelashes.

G. While the child is looking up, gently pull down the lower eyelid. Along the lower eyelid, squeeze out a line of ointment from the inner eye to the outer eye. When reaching the outer eye, rotating the tube will help detach the ointment from the line in the eyelid.

H. Have the child close his eyes for 1 to 2 minutes.

I. Gently wipe any excessive medicine from the eye while it is closed.

J. Before replacing the cap onto the tube, squeeze a small amount of ointment from the tube and discard it. This will help prevent contamination of the medicine.

SKIN MEDICINE

A. Wash your hands.

B. Wash the affected area of skin, unless otherwise directed by a physician, and pat dry with a clean towel.

C. Shake the liquid and aerosolized medicine.

D. Apply the medicine as directed by the physician. If no directions have been provided follow these general guidelines.

Creams, ointments, gels, solutions, and lotions: usually only a thin layer of the medicine needs to be rubbed onto the skin.

Aerosol: hold the canister at least 6 inches away from the skin and spray the affected area for several seconds.

E. Be careful not to get any medicine into the child's eyes, ears, or mouth.

F. Wash your hands after the medicine has been applied.

3. Placing inhaler in mouth

This is the least effective and least favorable way of giving medicine from an inhaler. It must be done only for children who do not have spacers and are not able to accurately squirt the medicine into their mouths from a distance.

a. Directions are the same as those for using an inhaler without a spacer (see item 1, Inhaler alone), except that here the inhaler is placed between the lips (follow steps b,c, d, and e).

E. If more than 1 puff is needed, wait at least 1 minute after the first puff before repeating steps a to d.

F. If multiple medicines from different inhalers are required, it is best to use an inhaled bronchodilator, like albuterol or metaproterenol, before any other.

Capsule Inhaler

A. Place the capsule properly into the inhaler.

Rotahaler: twist the back of the inhaler all the way to the right. Place the small end of the capsule into the opening on the back of the inhaler.

Spinhaler: pull up on the back of the inhaler and remove it. Firmly place the colored end of the capsule into the holder and replace the back of the inhaler.

B. Release the medicine from the capsule.

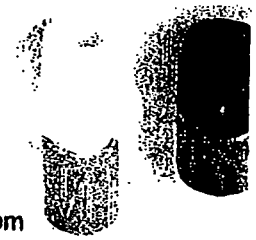
Rotahaler: twist the back of the inhaler all the way to the left.

Spinhaler: slide the grey sleeve all the way down and then back up.

C. Tell the child to breathe out completely.

D. Tilt her head back slightly and place her lips around the top of the inhaler.

E. Tell the child to inhale quickly and hold her breath for 10 seconds, or for as long as she can.



Left: Spinhaler which is used with cromolyn inhaled capsules (Intal).
Right: Rotahaler which is used with albuterol inhaled capsules (Ventolin Rotacaps).

F. Have the child remove the inhaler from her mouth and exhale.

G. Repeat steps C to F until no medicine remains in the inhal

H. Clean capsule inhaler by rinsing with water after each u and air drying.

Nebulizer

A. Add the prescribed volume of medicine and diluting liqu into the reservoir (usually a clear plastic cup located at tl end of the mouthpiece).

B. Turn the nebulizer on.

C. Be sure a mist is coming out of the mouthpiece befo placing it in the child's mouth.

D. Place the end of the mouthpiece into the child's mouth ar have the child breathe normally.

E. Unless otherwise directed, the child is finished when there is no more liquid in the reservoir.

F. Clean nebulizer after each use by rinsing the mouthpiece and reservoir with water. Once a week the mouthpiece and reservoir should be soaked in soapy water. Rinse and air dry after cleaning.

NASAL

Drops

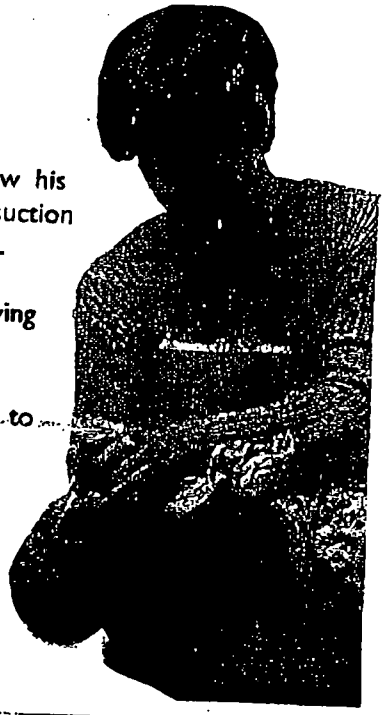
A. Have the child gently blow his nose if he is able; an infant suction bulb can be used for babies.

B. Wash your hands before giving the medicine.

C. Position the child according to the following guidelines:

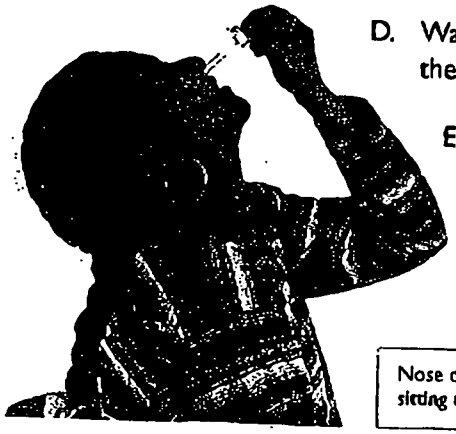
1. Place an infant lying down in your arms with his head tilted back.

2. If a child is too large to hold as described above, but is not able to sit upright for the entire procedure, have him lie on his back with a small pillow or other soft item between his shoulders. Then gently tilt his head back.



Nose drops being given to an infant. The infant is lying flat with her head tilted back.

3. Older children should sit in an upright position with their heads tilted back.



Nose drops being taken by a teenager. She is sitting upright with her head tilted back.

D. Warn the child that he may taste the medicine drops.

E. Warm the medicine to **room temperature** if it is cold.

F. Push up gently on the tip of the child's nose.

G. Draw up enough medicine into the dropper so the correct number of drops can be given.

H. Without touching the dropper to the nose, insert the dropper slightly (about one-third of an inch) into the nostril.

I. Instruct the child to breathe through his mouth while the medicine is being placed into his nose.

J. Aim the dropper toward the back of the nostril and squeeze out the prescribed number of drops.

K. Repeat steps F to J if the other nostril needs medicine.

L. After the correct number of drops has been given, tell the child to keep his head tilted back for 5 minutes. Allow him to spit out any medicine that runs down his throat.

M. If the child coughs, place him upright if he is not so. Keep him upright and watch for any problems with his breathing or for excessive coughing. If this occurs, call his physician or the Poison Control Center immediately.

Spray

A. Have the child gently blow his nose if he is able; an infant suction bulb can be used for babies.

B. Have the child sit upright with his head tilted back (as shown).

C. While plugging one nostril, place the tip of the sprayer slightly (about one-half of an inch) into the other nostril.

inner corner of the eye, have the child hold his breath.

E. While the child holds his breath, squeeze the sprayer quickly and firmly.

F. The child should continue to hold his breath for several more seconds.

G. Remove the sprayer from the child's nose and allow him to exhale through his mouth.

H. Repeat steps C to G if more sprays are prescribed, or if the other nostril needs medicine.

I. Instruct the child to keep his head tilted back for at least 2 minutes and avoid blowing his nose during this time.

J. Rinse the tip of the sprayer with warm tap water before replacing cap.

Aerosol

A. Have the child gently blow his nose if he is able; an infant suction bulb can be used for babies.

B. Shake the aerosol well and remove the protective cap.

C. Place the tip of the aerosol inside the child's nostril (as shown).

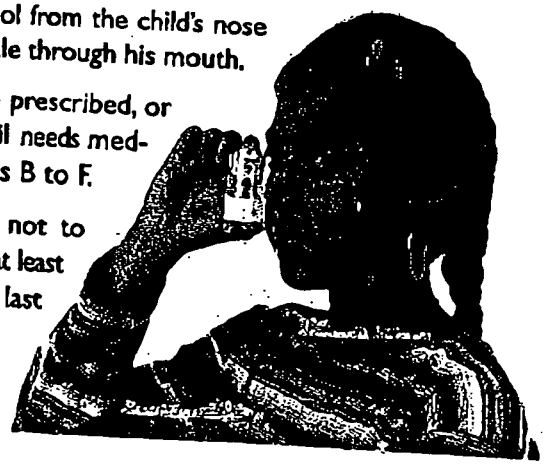
D. While the child holds his breath, firmly press down on the top of the aerosol and then release.

E. The child should continue to hold his breath for at least 3 more seconds.

F. Remove the aerosol from the child's nose and have him exhale through his mouth.

G. If more sprays are prescribed, or if the other nostril needs medication, repeat steps B to F.

H. Instruct the child not to blow his nose for at least 2 minutes after the last spray.



INSULIN INJECTION

A. Make sure the following items are ready to be used:

1. Sterile insulin syringe and needle
2. Vial(s) of insulin
3. Alcohol wipes or rubbing alcohol and gauze or cotton balls

B. Check the label on the insulin vial to be sure the correct type of insulin will be injected. (If the wrong type of insulin is given it increases the risk for too high or too low blood sugar.) Once the correct type of insulin has been confirmed, roll the insulin vial between your hands to mix the medicine. Do not shake the vial.

C. Clean the following areas with an alcohol wipe, gauze, or a cotton ball soaked in rubbing alcohol (do not use the same one to clean both areas):

1. Top of the insulin vial
2. The skin at the site where the injection will be given

D. Remove the protective cap from the needle of the syringe. Pull back on the plunger until the top of the black rubber stopper is at the same number that it will be at after the insulin is drawn into the syringe. This is done so that the same amount of air will be put into the vial that will match the amount of insulin taken out.

E. Place the insulin vial on a hard surface (such as a counter or table) and insert the needle through the rubber stopper of the vial.

F. Grab both the vial and syringe in one hand and turn them over. (Do not touch the needle.) Push on the plunger with the other hand until all the air is out, then pull it back to the prescribed dose of insulin to be injected (the top of the black rubber stopper should be in the same place as it was in step D).

G. Turn the vial and syringe back over and place the vial back on the hard surface. Pull the syringe out of the vial and turn it

over (the needle should be pointing up). Draw any insulin that is in the needle into the syringe by pulling back slightly on the plunger.

H. Examine the insulin inside the syringe. If there are any air bubbles, gently tap the syringe with your finger to move the air bubbles up to the needle. Once all the air bubbles are in the needle, carefully push up on the plunger until liquid just starts to come out of the tip of the needle. This removes all the air bubbles from the syringe and needle.

I. Reexamine the syringe. If any air bubbles remain, pull back on the plunger to put air into the needle and then repeat step H.

J. Pinch the skin at the injection site and hold it. Quickly insert the needle into the skin at a 90-degree angle.

K. Push down on the plunger to inject the insulin. Withdraw the syringe and dispose of it only in proper containers.

RECTAL

Suppository

A. Wash your hands.

B. Have the child lie on his left side; the left leg should be straight and the right leg should be bent up toward his chest.

C. If the suppository is soft, place it under cold running water before unwrapping until it becomes firm. Put on a glove and remove the suppository from its wrapper.

D. With one hand, gently separate the child's buttock cheeks so you can see the rectum.

E. With the other hand, quickly dip the suppository into some cool water or place a small amount of Vaseline or KY Jelly on the tip. Gently insert the smooth, rounded end of the suppository into the rectum.

F. With one finger (use your pinky finger for children younger than 3 years and your index finger for children older than 3 years and adults), push the suppository into the rectum (maximum distance of three inches) until there is no resistance.

G. Remove your finger and check to make sure the suppository is still in the rectum. If it has been inserted far enough the suppository should remain in place. If the suppository comes out, reinsert it into the rectum a little farther than before.

H. If the suppository remains in the rectum, hold the child's buttock cheeks together until the immediate urge to go to the bathroom has passed.

I. Have the child remain in the same position for about 20 minutes. If this becomes a problem, the child should at least sit or lie down for this amount of time, without going to the bathroom.

Ointment

A. Wash your hands and put on gloves.

B. Have the child lie on his left side with the top leg bent up toward his chest.

C. Place the applicator that comes with the ointment onto the end of the tube. Put a small amount of ointment or Vaseline onto the tip of the applicator.

D. With one hand, gently separate the child's buttock cheeks so you can see the rectum.

E. With the other hand, gently insert the applicator into the rectum. Once the applicator is inside, squeeze the tube to insert the prescribed amount of medicine.

F. Remove the applicator from the rectum and the ointment tube and clean it with soap and warm water.

G. Have the child remain in the same position for about 20 minutes. If this becomes a problem, the child should at least sit or lie down for this amount of time, without going to the bathroom.

Calculation of Medication Doses

In the health profession, the metric system is widely used. Milligrams, grams, or kilograms are used rather than ounces and pounds. Instead of cups or teaspoons for liquid volume, milliliters or liters are used. This section provides a brief introduction to the metric system and dosing of medication in children.

Only a few metric symbols will be needed to help you understand the dosing of medicine. These are summarized in Table 1.

Medicine doses (how much is to be taken) for children usually are calculated based on the child's weight. So, two children who are the same age but different weights may be given different doses of the same medicine.

Can two children of different weights receive the same dose of a medicine? Yes, for several reasons. First, there may not be an appropriate dosage form of the medicine available to provide the correct dose. For example, if a medicine comes as a 250-mg tablet and the dose by weight is 175 mg, the child probably will be given a dose of 250 mg.

Second, medicines have a dosage range, that is, a range in which a particular dose is appropriate. For example, a medicine may have a dosage range of 10–20 milligrams per kilogram of the child's weight (mg/kg). If a 10-kg child and a 20-kg child are each given 200 mg, both doses fall between 10 and 20 mg/kg.

Finally, some medicine doses are based on the "normal" weight of a child at a certain age. That is why some nonprescription medicines state, for example, that children 3–6 years of age receive one dose and children 7–12 years of age receive another. Even though actual weights of children may vary in these age groups, it has been found that an appropriate dose could be given to these groups based on the "normal" weight of children at these ages.

Table 1. Commonly Used Units for Weight and Volume

1 pound (lb)	=	2.2	kilograms (kg)
1 kilogram (kg)	=	1000	grams (g)
1 gram (g)	=	1000	milligrams (mg)
1 milligram (mg)	=	1000	micrograms (mcg)
1 teaspoon (tsp)	=	5	milliliters (mL)
1 tablespoon (tbsp)	=	15	milliliters (mL)
1 milliliter (mL)	=	1	cubic centimeter (cc)

Calculation of a dose

Let's take a close look at how you can determine if the child is receiving an appropriate dose of medicine based on the child's weight.

EXAMPLE:

A 44-lb child is given 200 mg of Medicine Z three times a day. The normal dosing range for Medicine Z is 20–40 mg/kg/day divided into equal doses given 3 times a day. How do you calculate the appropriate dose for this child?

The easiest way is by starting with the child's weight and the prescribed dose of the medicine.

1) Convert the child's weight from pounds to kilograms.

$$44 \text{ lb} \div 2.2 = 20 \text{ kg}$$

2) Add all the doses the child gets in one day to determine the total daily dose.

$$200 \text{ mg} \times 3 \text{ times a day} = 600 \text{ mg/day}$$

3) Divide the total daily dose by the child's weight in kilograms.

$$600 \text{ mg/day} \div 20 \text{ kg} = 30 \text{ mg/kg/day}$$

4) Compare the dose calculated in Step 3 to the normal dosing range.

$$\text{Step 3} = 30 \text{ mg/kg/day}$$

$$\text{Normal dosing range} = 20\text{–}40 \text{ mg/kg/day}$$

The dose determined in Step 3 is part of the normal dosage range; therefore, the dose given to the child is appropriate based on weight.

Another method of determining that the child is getting an appropriate dose is by starting with the child's weight and the dosage range of the medicine.

1) Convert the child's weight from pounds to kilograms.

$$44 \text{ lb} \div 2.2 = 20 \text{ kg}$$

2) Calculate the normal high and low daily doses.

$$\text{Low: } 20 \text{ mg/kg/day} \times 20 \text{ kg} = 400 \text{ mg/day}$$

$$\text{High: } 40 \text{ mg/kg/day} \times 20 \text{ kg} = 800 \text{ mg/day}$$

3) Divide the high and low daily doses by the number of times the child receives the medication.

$$\text{Low: } 400 \text{ mg/day} \div 3 \text{ times a day} = 133 \text{ mg/dose}$$

$$\text{High: } 800 \text{ mg/day} \div 3 \text{ times a day} = 266 \text{ mg/dose}$$

4) Compare the dose that the child receives with the calculated high and low doses to see if it falls within the range.

Child's dose: 200 mg 3 times a day

Low dose: 133 mg 3 times a day

High dose: 266 mg 3 times a day

The dose the child receives falls between the calculated high and low doses; therefore, the dose is appropriate based on the child's weight.

Not all medicine doses are based on weight; some are determined by calculating the body surface area (BSA) as measured in square meters (m^2). This is accomplished by using the following equation:

$$\text{BSA } (m^2) = \sqrt{\frac{\text{Height (inches)} \times \text{Weight (lb)}}{3131}}$$

Once the body surface area has been calculated, the appropriate dose or dosage range can be calculated by the same methods described above. The only difference in the calculation is that the child's body surface area is used in place of weight.

An example of calculating body surface area and a dose is provided below:

A 50-lb child who is 3'5" tall is to receive 100 mg/m^2/day of Medicine Z divided into equal doses given 3 times a day. How much medicine will this child receive with each dose?

1) Calculate body surface area of the child.

Weight: 50 lb

Height: (Must change 3'5" into inches to fit into the equation.) 3'5" = 41 inches

When a height of 41 inches and weight of 50 lb is placed into the equation, a body surface area of 0.81 m^2 is calculated.

$$0.81 \ (m^2) = \sqrt{\frac{41 \text{ inches} \times 50 \text{ lb}}{3131}}$$

2) Calculate the total daily dose of the medicine.

$$100 \text{ mg/m}^2\text{/day} \times 0.81 \text{ m}^2 = 81 \text{ mg/day}$$

3) Calculate the individual doses.

$$81 \text{ mg/day} \div 3 \text{ doses/day} = 27 \text{ mg/dose}$$

READING A MEDICATION PROFILE

Each medicine covered in this book is presented alphabetically in a standard format. We have tried to make it as easy to read and understand as possible. Below is an example of the format used and an explanation of the information in each section. See the example on pages 26 and 27.

① This is the generic name of the medicine. The generic name usually represents the active drug, or combination of drugs, that the medicine contains. There is only one generic name for each medicine.

② Unlike the generic name, a medicine may have many brand names. A brand name is simply the name the company has decided to call the medicine. Brand name products sometimes contain several different drugs. If a brand name product cannot be found in this book, check the listing of active ingredients on the product label.

③ This section states the common uses for which the medicines are prescribed and how they work.

④ This section provides the usual dose of the medicine and the number of times a day it is usually given. Both child and adult doses are provided, since many older children and adolescents may be given the adult dose.

⑤ Listed in this section are oral medications that may interact with the medicine you are giving to cause potentially serious or life-threatening problems. If a potential medication interaction is identified, the child's physician should be notified immediately so that appropriate actions can be taken. If you have any questions about these interactions, a pharmacist also should be able to answer your questions.

This is not a complete list of medication interactions. Always check with the child's physician or a pharmacist for a more extensive list of possible medication interactions.

⑥ The adverse side effects of the medicine are discussed in this section and are divided into two categories: minor and severe.

Minor adverse side effects are the less serious ones that may be observed in some people taking the medicine. Attention to minor adverse side effects is necessary since these can possibly be harmful. An example of this is sedation. This side effect is common with many medicines, but usually is considered to be minor. However, if the child is playing on th swing set while experiencing sedation from the medicine, she may b seriously injured by falling off the swing. Even though the side effect ma be minor, the child's physician should always be notified if one occurs

Severe adverse side effects are usually rare, but can be life-threatenin or be signs of a more serious problem. An example of this is unusu bruising or bleeding. This side effect may seem mild, but it may indicat that a condition called thrombocytopenia (low platelet count) ha developed. Platelets are used by the body to stop bleeding. If the platele count goes too low, the child could possibly bleed to death from minor injury. Even though these side effects do not occur very often, i adverse side effects designated as "severe" occur, the child's physicia should be notified as soon as possible.

⑦ This section discusses the proper storage and administration of th medicine. The answers to a variety of questions, such as whether th medicine should be refrigerated, crushed, mixed with fruit juice, o taken with food, can be found here.

⑧ The special instruction section contains directions that should be followed while the child is receiving the medicine, what may be done to prevent some common adverse side effects, and so on.

INHALED

Oral Inhaler

A. Remove the cap from the inhaler.

B. Hold the inhaler upright and shake it.

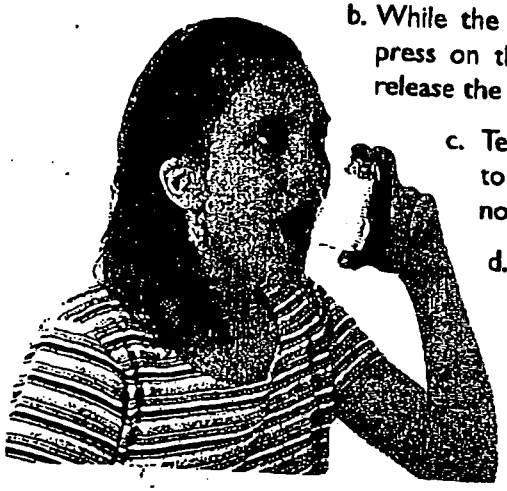
C. Tell the child to breathe out completely (blow out all the air).

D. Administer the medicine by one of the following methods.

E. Clean by soaking in soapy water once a week; rinse and air dry.

1. Inhaler alone

a. Position inhaler two or three fingers away from the mouth.



b. While the child slowly breathes in, press on the top of the inhaler to release the medicine.

c. Tell the child to continue to breathe in until she cannot breathe in any more.

d. Have the child hold her breath for 10 seconds or as long as she can hold it.

e. Have the child breathe out slowly.

2. Inhaler with a spacer

a. Place the inhaler into the spacer, and place the spacer into the child's mouth as shown on the facing page.

b. Press on the top of the inhaler to release the medicine into the spacer.

c. Tell the child to slowly breathe in and out several times, taking deep breaths and holding her breath for several seconds. If the spacer squeaks or whistles while the child is breathing, then she is breathing in or out too fast—tell her to breathe more slowly.

INHALER WITH A SPACER

A child using an Aerochamber with face mask. The mask should fit tightly over both the child's mouth and nose. The metallic medicine chamber is kept in its plastic holder and placed at the end of the spacer as shown.

A child using an Aerochamber with mouth piece. The metallic medicine chamber is kept in its plastic holder and placed at the end of the spacer as shown

A child using an Inspirease. The metallic medicine chamber is removed from its plastic chamber and placed at the top of the mouth piece as shown.

EXHIBIT B

Publication of Workshop Results

34th EMG EUROPEAN METABOLIC GROUP Meeting
Zurich, May 31st 2002 - June 2nd 2002

milupa
for Mother & Child

The 34th EMG EUROPEAN METABOLIC GROUP Meeting was held very successfully in Zurich from May 31st 2002 – June 2nd 2002. The scientific organisation for this year's main topic:

„Genetic Disorders of the Skeleton and of Connective Tissues"

was under the responsibility of Prof. Dr. Beat Steinmann and Prof. Dr. Andrea Superti-Furga, Zurich.

As in previous years, the results of the workshop which were held on the 1st of June 2002 are published in this booklet.

List of Contents

BH4-Responsive HPA/PKU

Chairpersons:

Nenad Blau (Zurich)
Ania Muntau (Munic)

BH_4-Responsive HPA/PKU

Chairpersons: Nenad Blau, Ania Mu[redacted]u

Introduction

BH_4 is an essential component of the phenylalanine hydroxylating system (Figure 1). BH_4-responsive hyperphenylalaninemia (HPA) or phenylketonuria (PKU) is a recently recognized variant of phenylalanine hydroxylase (PAH) deficiency. In particular patients with mild forms of PAH deficiency (plasma Phe < 1200 µmol/L) have been recognized to respond well to oral BH_4 administration . After a positive newborn screening revealing HPA, BH_4-responsive patients can be discriminated from BH_4-non responsive patients by a BH_4 loading test. The data so far available indicate that the incidence of BH_4-responsive PAH deficiency among individuals with mild HPA phenotypes is 70-80%. Genotypically, most of the patients with BH_4-responsive HPA show heterozygous mutations in the catalytic domain of the *PAH* gene. Some patients, however, bear homozygous mutations or mutations located in the regulatory or dimerization domains.

Phenylalanine hydroxylating system

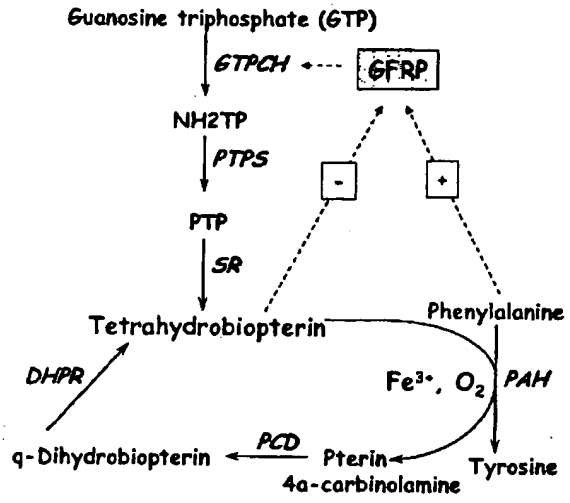


Figure 1. The phenylalanine hydroxylating system comprises apoenzyme phenylalanine hydroxylase (PAH), cofactor BH_4, and two regenerating enzymes pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR). While phenylalanine (Phe) activates the biosynthesis of BH_4 by induction of GTP cyclohydrolase I via the GFRP protein, BH_4 acts as a feed back inhibitor through the same mechanism.

In addition to four essential components (PAH, PCD, DHPR, BH_4) molecular oxygen and iron (III) are required for the fully coupled hydroxylation of phenylalanine to tyrosine. Iron is a part of the redox and reduction of Fe^{3+} to Fe^{2+} activates PAH. Phe is an allosteric effector (activator), while BH_4 negatively competes with Phe.

PAH mutations associated with BH_4-responsive HPA/PKU

The following *PAH* mutations have so far been reported to be associated with BH_4-responsiveness in patients with HPA/PKU due to phenylalanine hydroxylase deficiency: L48S, I65T, A104D, V190A, R241C, R261Q, A300S, A313T, A373T, E390G, A395P, A403V, P407S, Y414C. L48S and Y414C were found in homozygous states in single patients.

The mechanisms underlying BH_4-responsiveness in PAH deficiency are not yet understood. BH_4 may exert its effects by various mechanisms including :

1. Compensation for a reduced affinity of the enzyme for BH_4 (*K*-mutant)
2. Stabilization of the protein
3. Induction of *PAH* gene expression
4. Introduction of 3D structural changes in the PAH protein

Classification of HPA patients

So far, HPA patients have been classified according to pre-treatment plasma phenylalanine concentrations:

1. Classical PKU (pre-treatment plasma Phe ≥ 1200 µmol/L)
2. Mild PKU (pre-treatment plasma Phe 600 to 1200 µmol/L)
3. Mild HPA (Phe 120 to 600 µmol/L)
4. BH_4 deficiency (Phe > 200 µmol/L)

We propose a new clinical classification of HPA based on the feature of BH_4-responsiveness rather than on pre-treatment plasma phenylalanine concentrations:

1. BH_4-non-responsive HPA
2. BH_4-responsive HPA:
   a) BH_4-responsive PAH deficiency
   b) Defects in the BH_4 pathway.

Protocol for the standard BH_4 loading test for patients with plasma Phe > 400 µmol/L

Due to the marked [redacted]rogeneity in the response to BH_4, the s[redacted]vity of the test is increased significantly when an extended observation period is used. The revised protocol therefore recommends an observation period of 24 hours:

- The test is carried out after at least 3 hours of fasting
- Urine sampling for neopterin and biopterin before the beginning of the test
- Oral application of BH_4 (20 mg/kg bw)
- Continue food (Phe) intake during the whole test period
- Blood sampling for Phe and Tyr at 0, 4, 8, and 24 h
- Urine sampling for neopterin and biopterin at 4-8 h
- Blood spot for DHPR activity in erythrocytes (Guthrie card)

Protocol for the combined Phe-BH_4 loading test for patients with plasma Phe ≤ 400 µmol/L (mild HPA or child on diet)

- The test is carried out after at least 3 hours of fasting
- Urine sampling for neopterin and biopterin before the beginning of the test
- Oral application of Phe (100 mg/kg bw)
- Oral application of BH_4 (20 mg/kg bw) 3 hours after Phe load
- Continue food intake during the whole test period
- Blood sampling for Phe and Tyr at 0, 3, 7, 11, and 27 h
- Urine sampling for neopterin and biopterin at 7-11 h
- Blood spot for DHPR activity in erythrocytes (Guthrie card)

Definition of a positive loading test

Patients are classified as being BH_4-responsive when blood phenylalanine concentrations 24 hours after BH_4 challenge decrease by more than 30% of the value obtained before BH_4 administration. Some patients show a rapid decrease resembling that seen in patients with BH_4 synthesis defects, while others display a slow response reaching a maximum effect only 12 to 24 hours after cofactor application: Fast and slow responders.

Recent (unpublished) data:

In a large retrospective study (n=1939) the traditional BH_4 loading test was found to be positive in 65%, 74%, 33%, 17%, 0%, and 10% of patients with basal Phe levels of 120-400, 400-800, 800-1200, 1200-1600, 1600-2200, and >2200 µmol/L, respectively, when loaded with 20 mg 6R-BH_4/kg (C. Bernegger and N. Blau, unpublished results).

BH_4 was shown to significantly reduce blood phenylalanine concentrations and to enhance *in vivo* ^{13}C-phenylalanine oxidation in 27 out of 31 patients with mild HPA phenotypes, whereas 7 out of 7 patients with classical PKU did not respond to BH_4 (A.C. Muntau, unpublished results).

BH_4 (where to buy and how much to pay)

Schirck[redacted]oratories
Büchstrasse 10 - 8645 Jona - Switzerland
Tel.: +41 55 225 52 25
Fax: +41 55 225 52 26
e-mail: schircks@schircks.com

[redacted]	[redacted]	[redacted]
1,000	1.45	1,450.00
[redacted]	[redacted]	[redacted]
200	7.45	1,490.00

Long term BH_4 treatment

Data from a small pilot therapeutic trial in 5 children with mild PKU aged 4 to 14 years replacing dietary phenylalanine restriction by oral administration of BH_4 at daily dosages between 7.1 and 10.7 mg/kg body weight are now available. The duration of treatment was 207 ± 51.3 days (mean ± SD; range 166 - 263). Cofactor treatment led to a significant increase in the daily phenylalanine tolerance from 18.7 ± 8.6 mg/kg body weight (mean ± SD; range 8.5 - 30) before BH_4 treatment to 61.4 ± 27.9 mg/kg body weight (mean ± SD; range 17.9 - 90) on BH_4 treatment ($P < 0.05$) with little impact on the blood phenylalanine concentration (before treatment 366 ± 120 µmol/l, after treatment 378 ± 173 µmol/l; mean ± SD), A.C. Muntau, unpublished results.

At present no treatment recommendations can be given (experimental phase)!

- Multicenter trials will be initiated
  - Initial dosage 10 mg/kg x day
  - Individual titration/optimization
  - BH_4 + diet optional in PKU patients
- BH_4 is very expensive
  - Age 1mo diet (2,600 EUR) BH_4 (2,500 EUR)*
  - Age 2yr diet (3,000 EUR) BH_4 (7,000 EUR)*
  - Age 7yr diet (5,600 EUR) BH_4 (13,000 EUR)*

*10 mg/kg, all prices per year of treatment

References

Weekly updates at www.bh4.org

Steinfeld R, Kohlschütter A, Zschocke J, Lindner M, Ullrich K, Lukacs Z. Tetrahydrobiopterin monotherapy for phenylketonuria patients with common mild mutations. Eur J Pediatr 2002;DOI 10.1007/s00431-002-0966-0.

Lässker U, Zschocke J, Blau N, Santer R. Tetrahydrobiopterin responsiveness in phenylketonuria. Two new cases and a review of molecular genetic findings. J Inherit Metab Dis 2002;25:65-70.

Koch R, Güttler F, Blau N. Mental illness in mild PKU responds to biopterin. Mol Genet Metab 2002;75:284-286.

Blau N, Trefz FK. Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency: Possible regulation of gene expression in a patient with the homozygous L48S mutation. Mol Genet Metabol 2002;75:186-187.

Spaapen LJM, Bakker JA, Velter C, Loots W, Rubio ME, Forget PP, Duran M, Dorland L, de Konig TJ, Poll-The BT, Ploos van Amstel HK, Bekhof J, Blau N. Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency in Dutch neonates. J Iner Metabol Dis 2001;24:325-358.

Nuoffer JM, Thöny B, Romstad A, Blau N. A patient with phenylketonuria successfully treated with tetrahydrobiopterin. J Inerit Metab Dis 2001;24(Suppl. 1):29.

Erlandsen H, Stevens RC. A structural hypothesis for BH4 responsiveness in patiens with mild forms of hyperphenylalaninemia and phenylketonuria. J Inherit Metab Dis 2001;24:213-230.

Lindner M, Haas D, Zschocke J, Burgard P. Tetrahydrobiopterin responsiveness in phenylketonuria differs between patients with the same genotype. Mol Genet Metab 2001;73(1):104-6.

Trefz FK, Aulea-Scholz C, Blau N. Successful treatment of phenylketonuria by tetrahydrobiopterin. Eur J Pediatr 2001:315.

Leandro P, Rivera I, Lechner MC, de Almeida IT, Konecki D (2000) The V388M mutation results in a kinetic variant form of phenylalanine hydroxylase. Mol Genet Metab 69: 204-12

Kure S, Hou DC, Ohura T, Iwamoto H, S. S, Sugiyama N, Sakamoto O, Fuji K, Matsubara Y, Narisawa K. Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *Journal of Pediatrics* 1999;135(3):375-378.

SEROLOGICAL AND BIOCHEMICAL DATA

Serum	1979				1980			1981		1982	
	Feb. 12	Oct. 22	Oct. 31*	Dec. 17	March 19	July 1	Oct. 27	Feb. 9	Dec. 21	May 24	Dec. 27
HBsAg (ng/ml)	160 000	<0·2	<0·2	<0·2	130	+	650	300	30	10	30
HBsAb (mIU/ml)	–	5	3	–	–	–	–	–	–	–	–
HBcAb (reciprocal titre)	1000	1000	. .	1000	1000	. .	. .	1000	. .	1000	. .
HBeAg	–	–	–	–		. .	–	. .	. .		
HBeAb	+	+		+	+			+	. .	+	
Anti-δ antibodies (reciprocal titre)	. .	10	. .	. .	1000	. .	. .	100	. .	100	–
Bilirubin (mg/l)	. .	67	16	8	6	. .	. .	5		5	
Alanine aminotransferase (normal range 5–45 IU/l)	9	1,230	186	84	128	82	52	70	47	29	22
Alkaline phosphatase (normal range 40–200 IU/l)	110	788	530	140	300	249	122	102	81	69	64
γ-globulin (g/l)	8·5	13·1	12·2	15·0	10·0	12·4	11·5	11·3	12·0	11·8	12·7

Markers of HBV were detected by solid-phase radioimmunoassay (CNTS, Paris); anti-δ antibodies were measured according to Rizzetto et al.[δ]
*On Oct. 31, 1979, serum C3 and C4 levels were 175 and 25 mg/dl, respectively (normal range 80–160 and 20–40 mg/dl).

virus replication, whereas in chimpanzees which were positive for HBeAg the δ infection subsided rapidly.

INSERM Unit 56, 94270 Bicêtre, France — D. FROMMEL*, J.-P. ALLAIN
CNTS, Paris — A.-M. COUROUCE
INSERM Unit 45, UER Alexis Carrel, Lyon — S. DEROSE, D. TREPO
Ospedale di San Giovanni, Turin, Italy — O. CRIVELLI, M. RIZZETTO

*Present address: Hepatitis Immunovirology and Epidemiology Laboratory (INSERM Unit 45), UER Alexis Carrel, 69372 Lyon, France

LACK OF HYPERSENSITIVITY TO IONISING RADIATION IN FAMILIAL DYSAUTONOMIA

SIR,—It has been suggested that defective DNA repair may be involved in the pathogenesis of a range of neurodegenerative disorders. Tests of in vitro cell sensitivity to ionising radiation or other DNA damaging agents may give information about DNA repair mechanisms. These tests have now been done for several diseases of the nervous system, both familial and sporadic, as indicated by Dr Robbins and colleagues (Feb 26, p 468). Amongst the disorders reported by Robbins and colleagues as showing cellular hypersensitivity both to X-rays and the DNA alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine is the inherited neuronal disorder familial dysautonomia.[1,2] We have been unable to confirm the presence of increased radiosensitivity.

δ Rizzetto M, Shih JWK, Gocke DJ, Purcell RH, Verme G, Gerlin JL. Incidence and significance of antibodies to delta antigen in hepatitis B virus infection. *Lancet* 1978; ii: 986–90.
1 Robbins JH, Mcshell AN, Scarpinato SG, Tarone RA. Cells from patients with olivopontocerebellar atrophy and familial dysautonomia are hypersensitive to ionizing radiation. *Chn Res.* 1980; 28: 290A.
2. Scudiero DA, Meyer SA, Clatterbuck BE, Tarone RE, Robbins JH. Hypersensitivity to N-methyl-N'-nitro-N-nitrosoguanidine in fibroblasts from patients with Huntington disease, familial dysautonomia and other primary neuronal degenerations. *Proc Natl Acad Sci (USA)* 1981; 78: 6451–55.

RADIOSENSITIVITY OF FAMILIAL DYSAUTONOMIA AND CONTROL FIBROBLASTS

Dysautonomia			Controls		
Case	Age (yr)	D_0 (rad)	Case	Age (yr)	D_0 (rad)
GM 0732	1	148±17 *(6)*	A	1	124±4·0 *(3)*
GM 2341	17	171±0·3 *(2)*	B	11	109±1·5 *(3)*
GM 2342	19	141±3·5 *(3)*	C	15	192±4·2 *(3)*
GM 2343	24	146±10 *(8)*	D	30	159±9·0 *(3)*
			E	39	121±2·1 *(3)*
			F	41	160±4·3 *(4)*
Total		151±6·7 *(19)*			144±12 *(19)*

D_0 is the dose in rads which reduces survival of fibroblast clones from any point on the exponential part of the survival curve to 37% of the value at that point, and is given with standard error and (in parentheses) number of experiments. For experimental details see Chamberlain and Lewis.[3]

Fibroblasts from four patients with familial dysautonomia were obtained from the Institute for Medical Research, Camden, New Jersey, and clonal sensitivity to graded doses of ionising radiation was measured by standard techniques.[3] Calculated values for D_0 (in rads) showed no difference between familial dysautonomia and control cells (see table).

The pathogenesis of this disease is unknown, and the suggestion that defective DNA repair might be involved is attractive. Neuropathological studies show premature loss of neurons in autonomic ganglia and spinal sensory ganglia, with secondary changes in ascending spinal tracts and peripheral nerves; and spinal ganglion cell loss is a striking feature of the known or presumed DNA-repair-deficiency disorders xeroderma pigmentosum and ataxia telangiectasia, as well as in Friedreich's ataxia, where increased cellular X-ray sensitivity has been shown.[3] In their studies Robbins and his colleagues used virus-transformed lymphocyte cell lines,[1] the post-irradiation viability of which was determined by trypan blue dye-exclusion, while hypersensitivity to a chemical mutagen was indicated by work on fibroblasts.[2] Our study does not support the idea that DNA repair might be defective in familial dysautonomia and suggests the need both for caution in interpreting experiments suggesting abnormal cellular responses to DNA damaging agents and for multiple parallel experiments of the type devised by Teo and colleagues.[4]

Department of Histopathology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0HS — S. BRENNAN, P. D. LEWIS

SUCCESSFUL TREATMENT OF DEPRESSION WITH TETRAHYDROBIOPTERIN

SIR,—Certain subgroups of patients with endogenous depression seem to have a decreased activity of central serotonin and/or noradrenaline systems[1] and drug therapies directed at increasing noradrenaline and/or serotonin at synapses in the central nervous system have proved beneficial in certain cases. Tetrahydrobiopterin (BH_4), the co-factor for tyrosine and tryptophan hydroxylase, is thought to play an important role in regulating biogenic amine synthesis.[2] Co-factor content in cerebrospinal fluid (CSF) from depressed patients was first measured in 1978, although no

3. Chamberlain S, Lewis PD. Studies of cellular hypersensitivity to ionising radiation in Friedreich's ataxia. *J Neurol Neurosurg Psychiat* 1982; 45: 1136–38.
4. Teo IA, Arlett CF, Harcourt SA, Priestley A, Broughton BC. Multiple hypersensitivity to mutagens in a cell strain (46 BR) derived from a patient with immuno-deficiencies. *Mutation Res* 1983; 107: 371–86.
1. Goodwin FK, Cowdry RW, Webster MH. Prediction of drug response in the affective disorders. In: Lipton M, DiMascio A, Killam K, eds. Psychopharmacology a generation of progress. New York: Raven Press, 1978: 1277–88.
2. Levine RA, Kuhn DM, Williams AC, Lovenberg W. The influence of ageing on biogenic amine synthesis: The role of the hydroxylase cofactor. In: Raskin A, Robinson DS, Levine J, eds. Influence of age on the pharmacology of psychoactive drugs. Amsterdam: Elsevier, 1981: 37–45.

definitive conclusions were drawn.[3] We recently reported mood improvement in two depressed patients after a single oral dose of BH_4.[4] High-dose BH_4 treatment has also been shown to be effective in certain cases of BH_4-deficient hyperphenylalaninaemia,[5,6] Parkinson's disease,[7,8] and dystonia.[9] We now report an extended study of one chronically depressed patient, unmanageable by traditional antidepressive therapy, whose mood improved markedly concomitant with an increase in the CSF content of BH_4 and the biogenic amine metabolites 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA).

A 53-year-old female had not responded to either placebo or a wide variety of antidepressive drugs during 10 years of hospital care. (She had responded to 5-hydroxytryptophan [5-HTP] but long term use of this drug was not feasible because of the induction of manic symptoms uncontrollable by dosage adjustments.[10] Tryptophan alone had no effect.) The manic response to 5-HTP did indicate possible involvement of serotonin systems. 5-HTP caused severe nausea and vomiting that was not suppressed by addition of a peripheral decarboxylase inhibitor.

This patient was selected for further study from three patients who had been screened for elevation of mood after BH_4 given at a dose of 1 g by mouth in the early morning for 3 days; she was the only positive responder. BH_4 therapy induced intermittent vomiting, though not as severe that caused earlier by 5-HTP. On day 3, her mood had much improved. The improvement persisted for 3 days after BH_4 therapy was stopped, but then the patient deteriorated. Biochemical measurements indicated no correlation between mood and urinary free dopamine or serotonin.

For 2 months after the first trial, the patient was given no antidepressive drugs. Then BH_4 1 g daily (from Dr B. Schircks Laboratory, Schachenstrasse 4, Wettswill, Switzerland) was tried. After 5 days with no response, her diet was supplemented with tryptophan and tyrosine in case nausea and vomiting had been restricting her intake of these biogenic amine precursor aminoacids. On day 6 there was a marked clinical improvement that lasted over a week as determined by global assessment scales and the AMP rating method.[10] The elevation in mood on day 6 coincided with a significant increase in the CSF content of 5-HIAA, HVA, and biopterin. However, we do not know if CSF metabolites were increased in response to BH_4 before mood improvement. BH therapy was continued together with tryptophan (tyrosine w discontinued after 3 days) but the BH_4 dose was lowered to 500 m daily on day 8 and to 100 mg on day 13 in the search for an effectiv maintenance dose. When the patient was on 100 mg BH_4 daily h mood gradually deteriorated, though CSF 5-HIAA, HVA, ar biopterin concentrations were still high (table). Perhaps the metabolites are removed only slowly from the central compartme so that lumbar CSF values do not accurately reflect short-ter fluctuations in mood or brain biogenic amine metabolism; howeve basal metabolite levels were clearly elevated in response to BH BH_4 and tryptophan were discontinued on day 49, and on day 6 CSF 5-HIAA, HVA, and biopterin CSF had returned to the lo levels found before treatment.

CSF metabolite: biopterin ratios were fairly constant, indicatin that the rate of biogenic amine turnover may be direct proportional to brain BH_4 levels. The use of BH_4, both alone combination with aminoacid precursors or traditional therap should be considered a novel approach to antidepressive therapy

Department of Paediatrics, University of Zurich, Zurich, Switzerland — H.-CH. CURTIUS, A. NIEDERWIESE

Section on Biochemical Pharmacology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205, USA — R. A. LEVINE, W. LOVENBERG

Department of Psychiatry, University of Zurich — B. WOGGON, J. ANGST

3 Van Kammen DP, Levine RA, Sternberg D, Ballenger J, Marder S, Post R, Bunney, W. Preliminary evaluation of hydroxylase cofactor in human spinal fluid: Potential biochemical and clinical relevance in the study of psychiatric disease. *Psychopharmacol Bull* 1978, 14: 51–52.

4. Curtius H-Ch, Muldner H, Niederwieser A. Tetrahydrobiopterin: Efficacy in endogenous depression and Parkinson's disease. *J Neural Transmission* 1982; 55: 301–18.

5. Kaufman S, Kapatos G, McInnes RR, Schulman JD, Rizzo WB. Use of tetrahydropterins in the treatment of hyperphenylalaninemia due to defective synthesis of tetrahydrobiopterin: Evidence that peripherally administered tetrahydropterins enter the brain. *Pediatrics* 1982; 70: 376–80.

6. Niederwieser A, Curtius H-Ch, Wang M, Leupold D. Atypical phenylketonuria with defective biopterin metabolism. Monotherapy with tetrahydrobiopterin or sepiapterin, screening and study of biosynthesis in man. *Eur J Pediatr* 1982; 138: 110–12.

7. Curtius H-Ch, Niederwieser A, Levine RA, Muldner H. Therapeutic efficacy of tetrahydrobiopterin in Parkinson's disease. *Ad Neurol* (in press).

8. Narabayashi H, Kondo T, Nagatsu T, Sugimoto T, Matsuura S. Tetrahydrobiopterin administration of Parkinsonian symptoms. *Proc Japan Acad* 1982; 58: 283–87.

9. LeWitt PA, Newman RP, Miller LP, Lovenberg W, Eldridge R. Treatment of dystonia with tetrahydrobiopterin. *N Engl J Med* 1983; 308: 157–58.

10. Angst J, Woggon B, Schoepf J. The treatment of depression with L-5-hydroxytryptophan versus imipramine *Arch Psychiatr Neurol Sci* 1977; 224: 175–86.

INTESTINAL PERMEABILITY IN COELIAC DISEASE

SIR,—Dr Bjarnason and colleagues (Feb 12, p. 323) descril abnormal small-intestinal permeability to ^{51}Cr-labelled EDTA i coeliac patients successfully treated with a gluten-free diet. Dr S. (Ukabam and I have studied small-intestinal permeability in coeli patients on a gluten-free diet, using lactulose and mannitol a passive permeability markers. The degree of permeability abno mality was related to the degree of jejunal histological abnormalit and patients with apparently normal jejunal biopsy specimens ha normal small-intestinal permeability.[1]

Bjarnason et al. conclude that their study strongly suggested " persistent functional and/or structural abnormality of the sma intestine in coeliac disease" and that this could have aetiologic implications. These conclusions are wholly unwarranted from th data presented. To justify these conclusions, the jejunal specimer of the treated coeliacs would have had to be completely normal i every respect apart from permeability. However, the data presente suggest that this is not so. Five of the ten treated coeliac patients ha mucosal crypt ratios below the normal range and five had raise epithelial lymphocyte counts. These data should be interpreted a showing, at least in some cases, persisting functional and structur defects as a result of suboptimal jejunal response to a gluten-fr diet. Even if all the histological indices were normal, Bjarnason an

1. Cooper BT, Ukabam, SO. Changes in small intestinal permeability reflect the degre mucosal abnormality in coeliac patients on a gluten free diet. *Clin Sci* 1982; 63: 21

CSF 5-HIAA, HVA, AND BIOPTERIN CONTENT BEFORE, DURING, AND AFTER THERAPEUTIC TRIALS WITH BH_4

	Treatment			CSF concentration (pmol/ml)* of:			CSF ratios		
Day	BH_4 (mg)	Tryptophan (mg/kg	Tyrosine (mg/kg)	5-HIAA	HVA	Biopterin (B)	5-HIAA:B	HVA:B	Mood†
0	..	..	..	34± 6 *(6)*	143±12 *(6)*	12·7	2·70‡	11·3	–
6	1000	50	50	289± 5 *(6)*	648±18 *(6)*	51·0	5·67	12·7	++
16	100	50	..	147±14 *(5)*	270±27 *(5)*	25·7	5·72	10·5	+
20	100	50	..	319 *(2)*	590 *(2)*	55·8	5·72	10·6	–
63	..	..	..	60 *(2)*	116 *(2)*	10·8	5·60	10·7	–

*Number of analyses (of same sample on different days) shown in parentheses; values expressed as mean±SEM. 5-HIAA and HVA were analysed by high pressure liquid chromatograph (HPLC) with electrochemical detection; biopterin was measured after oxidation by HPLC with fluorescence detection.

†– = depressed; + = mild improvement; + + = dramatic improvement.

‡This deviant ratio may be explained by the very low CSF content of 5-HIAA, which approached the limit of assay sensitivity

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Therapeutic Efficacy of Tetrahydrobiopterin in Parkinson's Disease

*H.-Ch. Curtius, *A. Niederwieser, **R. Levine, and †H. Müldner

*Division of Clinical Chemistry, Department of Pediatrics, University of Zurich, CH-8032 Zurich, Switzerland; **Section of Biochemical Pharmacology, Hypertension-Endocrine Branch, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205; and †Landeskrankenhaus Wiesloch, D-6908 Wiesloch, West Germany

Tetrahydrobiopterin (BH_4) is the natural cofactor for phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase. It is thought that BH_4 plays an important role in regulating the *in vivo* activities of tyrosine and tryptophan hydroxylase, which are the rate-limiting enzymes in the biosynthesis of the catecholamines and serotonin, respectively. In Parkinson's disease, one of the predominant pathological manifestations is a premature degeneration of dopamine neurons that originate in the substantia nigra and terminate in the striatum. The loss of these nigrostriatal dopamine neurons leads to a relative lack of dopamine, which is commonly counteracted by the administration of the precursor of dopamine, L-dihydroxyphenylalanine (L-DOPA). Over the years, several investigators have suggested that the possible elevation of tyrosine hydroxylase activity in surviving dopamine neurons by BH_4 administration might provide an alternative means of elevating striatal synaptic dopamine. BH_4 crosses the blood-brain barrier very poorly, although it has recently been demonstrated that high doses of BH_4 administered to certain BH_4-deficient patients suffering from defective dihydrobiopterin biosynthesis (also referred to as hyperphenylalaninemia or atypical phenylketonuria) eliminates the need for biogenic amine neurotransmitter precursor support therapy (7). The use of BH_4 as the sole treatment for certain atypical PKU patients supports animal experiments (2) demonstrating that BH_4 can enter the brain when administered in high doses.

Large-scale clinical testing of BH_4 administration has posed certain problems. One hindrance to the therapeutic use of BH_4 in clinical trials is that pure BH_4 has only recently become available (8) and the compound is expensive. Nevertheless, we have administered BH_4 to 2 patients with Parkinson's disease to test the potential therapeutic efficacy of BH_4. Although our results are very preliminary, they indicate that BH_4 administration may be a useful form of therapy in certain cases of Parkinson's disease.

MATERIALS AND METHODS

Two patients with Parkinson's disease were selected to receive a single dose of BH_4. One patient was a 72-year-old female, the other was a 62-year-old male. Both patients had been treated with L-DOPA and bromocriptinmesilate (Pravidel from Sandoz) up to 2 days prior to BH_4 administration. After discontinuing therapy, hypokinesia, rigidity, and tremor were observed in both patients.

$BH_4 \cdot 2HCl$ was obtained from Dr. B. Schircks, Schachenstr. 4, CH-8907 Wettswil a.A. One gram $BH_4 \cdot 2HCl$ was mixed with 100 mg ascorbic acid in water and administered orally 1 hr before breakfast. The patients were observed over the course of the day for signs of clinical improvement.

RESULTS

Table 1 summarizes the effects that were observed after a single 1-g dose of BH_4 was administered to 2 parkinsonian patients. Between 4 and 5 hr after treatment, the symptoms of hypokinesia and rigidity disappeared completely in both patients, whereas their tremor was only partially improved. The beneficial effect of BH_4 lasted approximately 5 hr, at which time the prior clinical symptoms reappeared.

DISCUSSION

The concept of using the hydroxylase cofactor, BH_4, to treat patients with Parkinson's disease has emerged based on several recent observations. It was originally demonstrated (4,5) that the cofactor content was decreased by 50% in the cerebrospinal fluid of parkinsonian patients compared to age-matched controls, thus indicating a possible involvement of altered BH_4 metabolism in this disease. It was later shown (6) that biopterin content in postmortal striatal samples from parkinsonian brains was decreased by 80% compared with control values. It is unknown whether these observed decreases in cofactor content are secondary to dopamine neuronal loss or, alternatively, whether altered cofactor metabolism is an etiological factor in Parkinson's disease. In either event, we have provided preliminary evidence that BH_4 may be a useful therapy in Parkinson's disease.

It is of interest that the therapeutic benefit of BH_4 in Parkinson's disease was also independently observed by H. Narabayashi and co-workers *(this volume)*. They

TABLE 1. *Clinical response to tetrahydrobiopterin administration in patients with Parkinson's disease*

Symptoms	Patient 1	Patient 2
Hypokinesia	++	++
Tremor	+	+
Rigidity	++	++
Elevation of mood	+	+

(+) Partial improvement; (++) full improvement.

ODS

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was a 62-year-old male. Both
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noted a similar, mild effect after BH_4 administration to parkinsonian patients, which also had a short duration of action. In contrast to these reports, P. A. LeWitt and collaborators *(this volume)* administered BH_4 to parkinsonian patients and observed no clinical benefit in 2 patients. The nature of these differences is not clear; however, the study of LeWitt and co-workers used an intravenous bolus to administer BH_4, whereas oral administration was employed in the other studies yielding positive results. This may indicate that the time course of BH_4 entry into the brain is critical. Alternatively, not all patients may respond positively to BH_4 administration. Age of the patient and duration of the illness may be critical factors in the degree of patient responsiveness to BH_4. It is likely that younger patients with relatively short duration of the disease will have the best chance of responding.

Some interesting comparisons can be made between the use of BH_4 in atypical PKU and Parkinson's disease. Following the suggestion of Kaufman and co-workers *(personal communication)*, we also demonstrated that high dose BH_4 (10–20 mg/kg orally) was effective as the sole treatment in certain BH_4-deficient atypical PKU patients (7). Since biogenic amine neurotransmitter precursors were unnecessary with high doses of BH_4, this indicated that a sufficient amount of BH_4 was penetrating the blood-brain barrier. Surprisingly, the effect of a single oral dose of BH_4 lasted at least 4 days. However, the beneficial effect of BH_4 now reported in the 2 parkinsonian patients was of short duration (a few hours). This difference is probably explained by a fundamental difference in cofactor metabolism in these diseases. In BH_4-deficient hyperphenylalaninemia, there is a drastic reduction in the ability to synthesize BH_4. Thus, there is minimal, if any, BH_4 synthesized by biogenic amine neurons in the brain. As a result, BH_4 administration replaces the cofactor deficit and restores the BH_4-dependent hydroxylase activity. However, there is little evidence to suggest such a severe lack of BH_4 in Parkinson's disease. If altered cofactor metabolism is at all involved, the defect in BH_4 metabolism must be less severe than in atypical PKU. If the decrease in BH_4 content in Parkinson's disease is solely a reflection of dopamine cell loss, then the ratio of cofactor to tyrosine hydroxylase in surviving neurons might be the same as in a normal individual. Thus, BH_4 administration in Parkinson's disease would be aimed at elevating the activity of existing tyrosine hydroxylase molecules above and beyond the enzyme activity provided by endogenous BH_4. Some of the kinetic arguments supporting this approach (3) indicate that tyrosine hydroxylase may exist in multiple kinetic forms *in vivo* and a majority of enzyme molecules are in a less-active form and may be subsaturated with the endogenous concentration of BH_4.

The results of the present study, although preliminary, indicate the possible benefits of BH_4 administration in Parkinson's disease. It is clear that a large-scale clinical trial is necessary to ultimately determine the efficacy of BH_4 in Parkinson's disease. In a preliminary fashion, we have also tested the effectiveness of BH_4 administration in 3 patients with endogenous depression and found a dramatic improvement for several hours in the 2 patients diagnosed as inhibited endogenous depressives (1).

The short duration of BH_4 benefit in our studies with Parkinson's disease compared to atypical PKU indicate that higher brain cofactor concentrations may be necessary than can be obtained by high-dose BH_4 therapy, especially considering that BH_4 has been shown to be highly localized in striatal dopamine terminals where the concentration of BH_4 may be 100 μM or higher (3). It is possible that more lipophilic, active hydroxylase cofactors may achieve higher brain cofactor concentrations for a longer time period, which could enhance the effectiveness of cofactor administration in Parkinson's disease. We are currently investigating the ability of synthetic and active cofactor analogs to penetrate the blood-brain barrier. It is hoped that this approach may ultimately be even more effective than BH_4 administration for the treatment of Parkinson's disease and other diseases involving deficits of biogenic amine neurotransmitters.

ACKNOWLEDGMENTS

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REFERENCES

1. Curtius, H.-Ch., Müldner, H., and Niederwieser, A. (1982): *J. Neural Transm.*, 55:301–308.
2. Kapatos, G., and Kaufman, S. (1981): *Science*, 212:955–956.
3. Levine, R. A., Miller, L. P., and Lovenberg, W. (1981): *Science*, 214:919–921.
4. Levine, R. A., Williams, A. C., Robinson, D. S., Calne, D. B., and Lovenberg, W. (1979): In: *Advances in Neurology*, edited by L. J. Poirier, T. L. Sourkes, and P. J. Bedard, pp. 303–307. Raven Press, New York.
5. Lovenberg, W., Levine, R. A., Robinson, D. R., Ebert, M., Williams, A. C., and Calne, D. B. (1979): *Science*, 204:624–626.
6. Nagatsu, T., Yamaguchi, T., Kaio, T., Sugimoto, T., Matsuura, S., Akino, M., Nagatsu, I., Iisuka, R., and Naraboyashi, H. (1981): *Clin. Chim. Acta*, 109:305–311.
7. Niederwieser, A., Curtius, H.-Ch., Wang, M., and Leupold, D. (1982): *Eur. J. Pediatr.*, 138:110–112.
8. Schircks, B., Bieri, J. H., and Viscontini, M. (1977): *Helv. Chim. Acta*, 60:211–214.

EXHIBIT E

United States Patent [19]

Curtius et al.

[11] Patent Number: 4,774,244

[45] Date of Patent: Sep. 27, 1988

[54] USE OF PTERIN DERIVATIVES

[75] Inventors: Hans-Christoph Curtius, Zollikon, Switzerland; Heinrich-Georg Müldner, Weinheim, Fed. Rep. of Germany; Alois Niederwieser, Pfaffhausen, Switzerland

[73] Assignee: Kanagafuchi Chemical Industry Company, Limited, Kitaku, Japan

[21] Appl. No.: 149,118

[22] Filed: Jan. 27, 1988

Related U.S. Application Data

[60] Division of Ser. No. 18,789, Feb. 20, 1987, which is a continuation of Ser. No. 775,162, Sep. 12, 1985, abandoned, which is a continuation of Ser. No. 471,287, Mar. 2, 1983, abandoned.

[30] Foreign Application Priority Data

Mar. 3, 1982 [CH] Switzerland 1300/82

[51] Int. Cl.[4] A61K 31/50; A61K 31/495

[52] U.S. Cl. 514/249

[58] Field of Search 514/249

Primary Examiner—Stanley J. Friedman

Attorney, Agent, or Firm—Ladas & Parry

[57] ABSTRACT

L-erythro-5,6,7,8-tetrahydrobiopterin, L-sepiapterin, 1',2'-diacetyl-5,6,7,8-tetrahydrobiopterin and 6-methyl-5,6,7,8-tetrahydropterin can be used for the therapeutic treatment of patients with Parkinson's disease and of patients with depression.

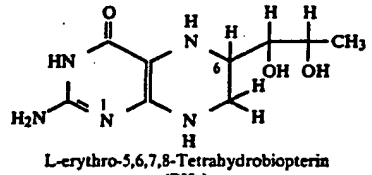
1 Claim, No Drawings

USE OF PTERIN DERIVATIVES

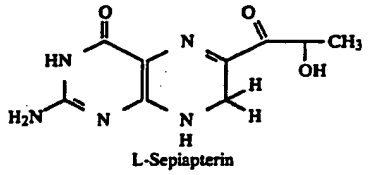
This is a divisional of co-pending application Ser. No. 018,789 filed on Feb. 20, 1987 which is a continuation of application Ser. No. 775,162 filed Sept. 12, 1985 (now abandoned), which in turn is a continuation of application Ser. No. 471,287 filed Mar. 2, 1983 (now abandoned).

It was known that L-erytho-5,6,7,8-tetrahydrobiopterin is the natural cofactor of phenylalanine 4-hydroxylase (EC 1.14.16.1), tyrosine 3-hydroxylase (EC 1.14.16.2) and tryptophane 5-hydroxylase (EC 1.14.16.4) [Massey V., Hemmerich P. in The Enzymes (Editor: Boyer PD), 3rd edition, volume 12, pages 191-252, Academic Press. Inc., New York 1975]. The latter two are the key enzymes for the biosynthesis of the neuro transmitters dopamine and serotonin.

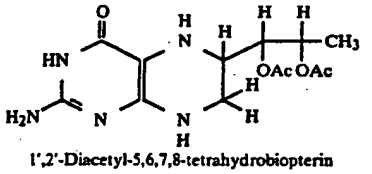
It has now been found, surprisingly, that L-erythro-5,6,7,8-tetrahydrobiopterin (abbreviated to BH_4 in the following text), sepiapterin, 1',2'-diacetyl-5,6,7,8-tetrahydrobiopterin and 6-methyl-5,6,7,8-tetrahydrobiopterin are effective for treating patients with Parkinson's Disease and patients with depression.



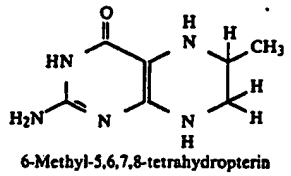
L-erythro-5,6,7,8-Tetrahydrobiopterin (BH_4)



L-Sepiapterin



1',2'-Diacetyl-5,6,7,8-tetrahydrobiopterin



6-Methyl-5,6,7,8-tetrahydropterin

All patients with depression had previously been treated with commerically available antidepressants and, in some cases, also with various neuroleptic agents. Some of the patients had not responded to the medicaments administered; the clinical picture had remained unchanged. After oral administration of one gram of BH_4 (stabilized against oxidation by the addition of 100 mg of ascorbic acid) in orange juice, a prompt improvement in the clinical picture of severe depression occurred after about 4–5 hours.

Others of the patients had likewise previously been treated with antidepressants. These patients had responded positively to individual antidepressant medicaments, as is shown in wide clinical experience. As usual, the action had its onset in the period from one to three weeks. After discontinuation of this medication, the old condition recurred. In contrast to conventional antidepressant treatment, when BH_4 was then administered in the manner described above, a prompt improvement in the clinical state occurred within a few hours (4–5 hours).

No side effects were observed. The side effects which are customarily observed on initial administration of antidepressants and neuroleptics (of an autonomic and extrapyramidal nature) never occurred. After discontinuation of BH_4, the old clinical state recurred within 12 to 16 hours.

CASE DESCRIPTIONS

In the first place, the symptoms of endogenous depression of the inhibited depression type will be described:

1. Lack of motivation (lack of initiative to carry out mental tasks and motor actions with slowing down of movement and thought processes)
2. Lack of concentration
3. Affective apathy (inactivity, inability to feel grief)
4. Feelings of insufficiency, particularly in the morning
5. Severe loss of weight
6. Insomnia.

(1) Man aged 29 with endogenous depression (familial affliction), above symptoms 1 to 6 very pronounced, very severe loss of weight; a number of depressive phases which were not treated. Dissociative lapses and increased risk of suicide during the duration of the disease. Administration of BH_4 without previous antidepressant treatment; symptoms 1, 2 and 3 were virtually abolished or no longer detectable 4–5 hours later. No statement can be made about symptoms 4, 5 and 6, since their disappearance would only have been observed after a longer time.

(2) Man aged 43 with endogenous depression, mother a chronic depressive, symptoms as described in (1), also feelings of guilt. Hospitalized for 10 years and treated with antidepressants, but with inadequate success. Prompt improvement in the state in the form mentioned after administration of BH4.

The patients with Parkinson's disease who were investigated had previously been treated with L-dopa and bromocriptine mesilate (Parlodet, Pravidel) and partial compensation of the clinical picture had been achieved. The onset of action with this treatment was observed after about 6 days. Using BH_4, not only was the onset of action considerably more rapid but almost complete compensation of the existing complaints was achieved.

CASE DESCRIPTIONS

The three symptoms characteristic of Parkinson's disease are akinesia (slow and incomplete movements which give rise to the impression of a decreased motor initiative), tremor (which becomes less intense on movement or maintaining a position and more intense with emotional excitement) and depression (in the form of affective instability).

(1) Woman aged 72 with idopathic Parkinsonism (familial affliction) had the symptoms mentioned. The three groups of symptoms had virtually disappeared about 4–5 hours after oral administration of BH_4, and

this is a result which had not been observed even after treatment with L-dopa.

(2) The same course was observed after treatment with BH_4 of a man aged 62 with idiopathic Parkinsonism.

The action was also demonstrated biochemically. For example, the concentrations of biopterin, dopamine and serotinin which are shown in Table 1 were found in the urine of two patients with an evident endogenous depression after administration of 1 g of tetrahydrobiopterin.2HCl(BH_4) or 0.9 g of diacetyltetrahydrobiopterin.2HCl.

TABLE 1

Biopterin, dopamine and serotonin in the urine of a patient with inhibited depression after oral administration of tetrahydrobiopterin or diacetyltetrahydrobiopterin respectively.

Urine collection at time (hrs)	Concentration after 1 g of tetrahydrobiopterin.2 HCl			Concentration after D,9 g of 1',2'-diacetyltetrahydrobiopterin.2 HCl		
	Biopterin mmol/mol Creatinin	Dopamin µmol/mol Creatinin	Serotonin	Biopterin mmol/mol Creatinin	Depamine µmol/mol Creatinin	Serotonin
0	0.36	147	30	0.90	78	24
2	1.36	208	42	0.77	180	34
4	6.71	220	60	1.13	200	57
8-10	6.04	220	40	—	—	—
12	0.91	210	34	0.82	58	61
Normal range	0.31-1.09	70-170	22-60	0.31-1.09	70-170	22-60

The increase in the biopterin concentration in the urine shows that the administered tetrahydrobiopterin is at least partially absorbed and that the ester groups in the diacetate can be endogenously hydrolyzed. It is seen from the table that the initial figures for the two neurotransmitters were sometimes in the lower part of the normal range and that they increased under treatment with tetrahydrobiopterin or diacetyltetrahydrobiopterin. This was paralleled by the clinical improvement observed.

Treatment of patients with Parkinson's disease or with depression can start with an initial dose of the order of 1 g/day (about 15 mg/kg of body weight) and be continued, for example, with a dose of 500 mg (about 7.5 mg/kg) on the second day and of 200 mg/day (about 3 mg/kg) from the third day.

What is claimed is:

1. A method for the therapeutic treatment of patients with depression which comprises orally administering to the said patients an effective amount of L-erythro-5,6,7,8-tetrahydrobiopterin, L-sepiapterin, 1',2'-diacetyl-5,6,7,8-tetrahydrobiopterin or 6-methyl-5,6,7,8-tetrahydrobiopterin.

* * * * *

Acta Neurol. [redacted]., 1989:79:493–499

Key words: Parkinson's disease; biopterin; dopamine; serotonin; HVA; 5-HIAA; tyrosine; tryptophan; phenylalanine.

Tetrahydrobiopterin and Parkinson's disease

I. C. Dissing[1], F. Güttler[1], H. Pakkenberg[2], H. Lou[1], A.-M. Gerdes[1], C. Lykkelund[1], V. Rasmussen[1]

[1]John F. Kennedy Institute, Glostrup, [2]Department of Neurology, Hvidovre Hospital, Denmark

ABSTRACT - Two patients with Parkinson's disease were treated with 1 g tetrahydrobiopterin (BH_4) for 5 days. Clinical improvement was not observed. In the cerebrospinal fluid (CSF) a 4–8 fold increase in the concentration of homovanillic acid (HVA), and a 3-fold increase in the concentration of 5-hydroxyindole acetic acid (5-HIAA) was measured. However, the concentration of HVA reached, was only approximately half as high, as that of patients treated with madopar (DOPA + benserazid). In urine, the excretion of HVA increased 13–37 fold, when the patients were treated with madopar, whereas no increase in the HVA excretion was measured after the BH_4 administration. Additionally, 2 patients with Parkinson's disease were treated with 1 g BH_4 in combination with 15 g tyrosine for 3 days, and 1 parkinsonian patient was treated with 15 g tyrosine daily for 7 weeks. No increase in the CSF concentrations of HVA or 5-HIAA was observed. The results suggest, the BH_4 in the dosage used, is not effective in the treatment of Parkinson's disease.

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Tetrahydrobiopterin (BH_4) is the natural co-factor of 3 important amino acid hydroxylases: tyrosine hydroxylase (EC 1.14.16.2), the rate-limiting enzyme in the biosynthesis of dopamine from tyrosine (1), tryptophan hydroxylase (EC 1.14.16.4), the ratelimiting enzyme in the biosynthesis of serotonin from tryptophan (2), and phenylalanine hydroxylase (EC 1.14.16.1), the enzyme catalyzing the conversion of phenylalanine to tyrosine (3). Phenylalanine hydroxylase is the deficient enzyme in phenylketonuria.

BH_4-deficiency can be caused by deficient activity of dihydropteridine reductase (DHPR) (EC 1.6.99.7) (4), the enzyme catalyzing the regeneration of BH_4, or by a defect in one of at least 3 enzymes essential for its biosynthesis (Fig. 1) (5, 6). BH_4-deficiency is a rare condition with an estimated incidence of 1% in newborns with hyperphenylalaninaemia (7). Because patients with BH_4-deficiency in addition to an impaired ability to convert phenylalanine, suffer from defects in the biosynthesis of the neurotransmitters dopamine, serotonin, norepinephrine, and epinephrine (8, 9), a low phenylalanine diet is not sufficient to prevent the development of severe neurological dysfunction and mental retardation in these patients. Treatment has to be supplemented with the amine precursors L-dopa and 5-hydroxytryptophan in combination with an inhibitor of peripheral aromatic amino acid decarboxylation or, the administration of a synthetic BH_4-derivative, to patients on a normal phenylalanine unrestricted diet (10, 11).

In Parkinson's disease the main characteristic biochemical change in the post-mortem brain is a greatly reduced concentration of dopamine in the basal ganglia and the substantia nigra (12, 13, 15). But in addition, the concentration of dopa-

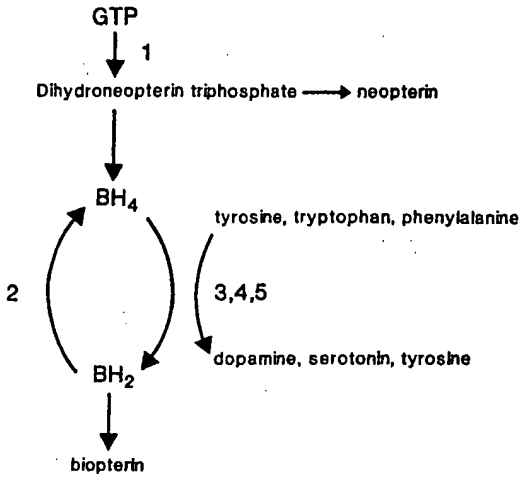

Fig. 1. A simplified illustration of the metabolism of BH_4. GTP: Guanosine triphosphate. 1: GTP-cyclohydrolase 1. 2: Dihydropteridine reductase (DHPR). 3: Tyrosine hydroxylase. 4: Tryptophan hydroxylase. 5: Phenylalanine hydroxylase.

mine, serotonin, and norepinephrine is reduced, though to a lesser extend, in almost all investigated parts of the brain (13, 14, 15). Reduced concentrations of dopamine and serotonin in the brain is reflected by reduced concentrations of the metabolites homovanillic acid (HVA) and 5-hydroxyindole acetic acid (5-HIAA) in the cerebrospinal fluid (CSF) (16, 17, 18).

Administration of L-dopa to patients with Parkinson's disease causes improvement in most, presumable by increasing the concentration of dopamine in the brain (15, 19). When BH_4 became available for the treatment of BH_4-deficiency, it was soon after considered, whether BH_4 could likewise be an alternative drug in the treatment of Parkinson's disease.

Furthermore, the BH_4 co-factor seems somehow to be involved in the pathology of Parkinson's disease. Thus, the concentration of BH_4 is reduced in the CSF of patients with Parkinson's disease (20), and the total concentration of biopterin, a metabolite of BH_4 (Fig. 1), is reduced in the post-mortem caudate nucleus (21).

In addition, the potent neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), which has been suggested as a possible etiologic agent in Parkinson's disease, because it induces a very similar clinical and neuropathological condition in humans, monkeys and some laboratory animals, has been found to be an inhibitor of DHPR (Fig. 1) (22).

The purpose of this study was to examine the clinical effect of BH_4 in Parkinson's disease, and to measure the concomitant biochemical changes in CSF, plasma, and urine.

The clinical and the biochemical changes produced by the administration of BH_4 in combination with the dopamine precursor tyrosine, and by tyrosine alone, were also investigated.

Table 1
CSF-concentrations of neurotransmitter metabolites, pteridines and amino acids in Parkinsonian patients before and during treatment with BH_4

Patient	Treatment	HVA nmol/l	5-HIAA nmol/l	Biopterin nmol/l	Neopterin nmol/l	Tyrosine umol/l	Tryptophan umol/l	Phenylalanine umol/l
	0 *	51.4	29.2	4.9	13.0	7.1	2.1	10.1
1.	BH_4	187.1	96.6	8.0	13.1	6.6	1.8	8.6
	Madopar	323.0	42.0	3.8	10.8	9.8	1.8	9.9
	0	19.3	34.6	3.9	12.4	8.7	2.7	12.9
2.	BH_4	161.9	106.4	8.6	19.2	6.3	1.8	9.7
	0	42.9	59.2	3.6	11.2	8.3	2.3	12.4
	BH_4 + tyrosine	49.4	47.1	7.4	10.6	15.6	2.5	13.6
3.	0	54.4	31.4	6.1	10.9	8.4	2.6	16.4
	BH_4 + tyrosine	60.6	47.1	11.3	9.3	22.1	3.2	14.0
	Tyrosine	64.1	40.1	6.3	13.6	17.2	2.7	15.6
Normal Controls	Median	193	96	10.4	10.8	7.7	1.8	8.1
	Range	(104–332)	(55–213)	(5.0–26.4)	(8.1–18.9	(6.6–13.0)	(1.3–2.8)	(5.9–10.4)

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	14.0
	15.6
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.8)	(5.9–10.4)

Material and methods

BH_4 (6R, S)-5, 6, 7, 8-tetrahydro-L-biopterin dihydrochloride was obtained from Dr. B. Schirks Laboratories, Switzerland. Tyrosine was obtained from Jensen Clinical Nutrition Service, Denmark. Permission to carry out the study was obtained from the Municipal Committee of Ethics, and informed consent was obtained from the patients.

Patients 1. A 46-year-old Caucasian man who had had Parkinson's disease for 14 years. He was treated with the usual anti-Parkinson drugs, but his condition remained very unstable with many on/off periods, and violent hyperkinesia daily (Hoehn & Yahr scale = 3–4).

Patient 2. A 43-year-old Caucasian man with Parkinson's disease of 5 years duration. Treatment with classical anti-Parkinson drugs produced a good response. No hyperkinesia and no on/off phenomena. (Hoehn & Yahr scale = 2).

Patient 3. A 40-year-old man not previously treated for Parkinson's disease. For 12 months he had experienced gradually progressing gait difficulty and problems in changing position in bed. He presented with light to moderate universal oligokinesia, rigidity and slight resting tremor of the hands.

Normal controls. Ten otherwise healthy patients with lower back pain served as controls for the CSF values, as they were having a diagnostic myelography. Exclusive criteria were a total protein concentration in the CSF beyond the normal range. The median age of the control group was 46 years, the range 22–64 years. The median age of the Parkinsonian group was 43 years, the range 40–46 years.

Treatment. Patients 1 and 2 received treatment with BH_4 alone. They were admitted to hospital and received usual anti-Parkinson medication (DOPA + benserazid) for 3 days, no medication for 5 days, and BH_4 for 5 days. The treatment was performed as an open study.

Additionally, Patients 2 and 3 received treatment with BH_4 in combination with tyrosine. Again, the patients were admitted to hospital. Patient 2 received usual anti-Parkinson medication for 3 days, no medication for 3 days, and BH_4 in combination with tyrosine for 3 days. Patient 3, who had not previously been treated, received no medication for 3 days and BH_4 in combination with tyrosine for 3 days, and, finally, tyrosine alone for 7 weeks.

BH_4 was administered to the patient at 08.00, before breakfast, in a 100 ml clear solution of 1 g BH_4, 1 g ascorbic acid, and 1 g sugar. Tyrosine was administered to the patients 3 times a day, at meals, as 10 tablets, each containing 0.5 g tyrosine.

Clinical examinations. During the experimental periods, the patients were examined using the Webster rating scale 3 times a day (8 a.m., 10.30 a.m., and 6 p.m.).

Table 2 *
Urine concentrations of neurotransmitter metabolites and pteridines in Parkinsonian patients while treated with Madopar and before and during treatment with BH_4

Patient	Treatment	HVA umol/ mol creat	5-HIAA mmol/ mol creat	Biopterin mmol/ mol creat	Neopterin mmol/ mol creat
	Madopar	67.2	1.6	0.59	0.24
1.	0	1.8	1.9	0.52	0.24
	BH_4	2.1	2.2	5.28	0.38
	Madopar	17.5	0.7	0.41	0.33
2.	0	1.4	2.0	0.43	0.28
	BH_4	1.3	1.5	3.15	0.30

CSF samples. Lumbar puncture were standardized with the patients in bed overnight, and until 10.30 a.m., when the punctures were taken. The CSF was collected in 5 fractions of 1 ml, immediately frozen, and stored at -80°C until analyzed by HPLC. The concentrations of HVA, 5-HIAA, biopterin, and neopterin was determined by use of electrochemical detection (23, 24), those of tyrosine, tryptophan, and phenylalanine, by use of fluorometric detection (24) (coefficient of variation better than ±5%). The mean of the 5 fractions were calculated. The lumbar punctures were done on the last day of each period of no-treatment, after 5 days of treatment with BH_4 alone, after 3 days of treatment with BH_4 in combination with tyrosine, and after 7 weeks of treatment with tyrosine alone. For comparison, Patient 1 had a lumbar puncture, while being treated with Madopar, in a dose sufficient to cause optimal relief of the Parkinsonian symptoms (600 mg DOPA).

Blood samples. Venous blood was drawn on the last day of each treatment period. When BH_4 was administered alone, blood samples were obtained 3 times daily, at 8 a.m., 10.30 a.m., and 1.00 p.m. When BH_4 was administered in combination with tyrosine, and when tyrosine was administered alone, blood samples were obtained at 10.30 a.m. The blood was immediately heparinized, frozen, and stored at -20°C, until analyzed by the HPLC for its concentration of tyrosine, tryptophan, and phenylalanine (24).

Urine samples. The urine was collected over 24 h on the same days as the blood samples were taken. The urine was immediately stored at -20°C in a solution of 10 ml 1 N HCl and 0.1 g cystin pr. 2 h sample period until analyzed by the HPLC for its concentrations of HVA, 5-HIAA, biopterin, and neopterin (23, 24).

Results

The Webster scores for Patients 1 and 2 were almost constant during the first 3 days of normal treatment. During the following 5 days without treatment, the Webster score increased constantly, and during the 5 days of BH_4 treatment there was no change in Webster score. Clinically, Patient 1 felt that his state was unchanged, Patient 2 felt that he improved a little bit.

Patient 2 reacted similarly during the experiment with BH_4 in combination with tyrosine: there was no improvement in the treatment phase, the Webster scores were unchanged.

Patient 3 experienced a subjective improvement on BH_4 in combination with tyrosine, but the Webster score did not confirm this. Tyrosine alone, had no effect.

Compared with normal controls, the patients with Parkinson's disease had, when untreated, reduced concentrations of HVA and 5-HIAA in the CSF. The concentration of biopterin was reduced in the CSF of Patients 1 and 2. The concentration of phenylalanine tended to be increased, whereas the concentrations of neopterin, tyrosine, and tryptophan were within the normal range (Table 1).

Administration of BH_4 produced a 4 to 8-fold increase in the CSF concentration of HVA and a 3-fold increase in the CSF concentration of 5-HIAA. The concentration of biopterin increased approximately 2-fold. There was a slight increase in the CSF concentration of neopterin and a slight decrease in the CSF concentration of the amino acids tyrosine, tryptophan, and phenylalanine (Table 1).

When BH_4 was administered in combination with tyrosine an approximately 2-fold increase in the CSF concentrations of biopterin and tyrosine was found, but this was not followed by changes in the CSF concentration of HVA and 5-HIAA (Table 1).

Administration of tyrosine alone produced a 2-fold increase in the CSF concentration of tyrosine, but no changes in the CSF concentrations of biopterin, HVA or 5-HIAA (Table 1).

Treatment with Madopar produced a 6-fold increase in the CSF concentration of HVA (Table 1).

The plasma concentrations of tyrosine, tryptophan and phenylalanine varied greatly during the day. No changes were observed with BH_4. Administration of tyrosine alone, or in combination with BH_4, produced an approximately 2-fold increase in the concentration of tyrosine.

BH_4 alone, or in combination with tyrosine produced a 8 to 11-fold increase in the concen-

tration of biopterin in urine, but this was not followed by increases in the concentrations of neopterin, HVA, or 5-HIAA (Table 2).

No changes were found in the concentration in urine of any of the measured parameters after the administration of tyrosine alone.

Discussion

The effects of BH_4 on Parkinson's disease has been studied, but with conflicting results, and because BH_4 is expensive, the number of patients has been small.

Thus, clinical improvement was found in 5 patients, with a single dose of 300–600 mg BH_4 (25) and in 2 others with a single dose of 1 g BH_4 (26), whereas no clinical improvement was found in 4 previously untreated patients, who received 200 mg BH_4 daily for 3 days (27).

When BH_4 was administered intravenously, no clinical improvement was found in 2 Parkinson's patients, who received between 2.5 and 10 mg BH_4 per kg body weight (28), whereas clinical improvement was found in 3 with foot-dystonia, otherwise responsive to L-dopa treatment, who received between 1.5 and 5 mg BH_4 per kg body weight (29).

The present study investigated the effect of 1 g BH_4 in only 2 patients, because no clinical improvement was found. It was however shown, that the administration of BH_4 produced a substantial increase in the concentration of HVA (4–8 times) and 5-HIAA (3 times) in the CSF. Since the concentration of HVA in the CSF of Patient 1 was nearly twice as high when the patient was treated with Madopar, compared with BH_4, it may be that the dosage of BH_4 was too small.

That the administered BH_4 was crossing the blood-brain barrier was indicated by the 2-fold increase in the CSF concentration of biopterin, as well as by the increase in the CSF concentrations of HVA and 5-HIAA.

The slight increase in the CSF concentration of neopterin after BH_4 administration may have been due to a slight feed-back inhibition of the natural biosynthesis of BH_4 and the slight decrease in the CSF concentration of tyrosine, tryptophan and phenylalanine, to a sligthly increased metabolism of these amino acids.

Untreated patients with phenylketonuria have reduced concentrations of HVA in CSF (30). Administration of tyrosine to these patients produces an increase in the concentration of HVA in the CSF (31), and administration of tyrosine to rats produces an increase in the concentration of dopamine in the brain (32). In order to try to produce a further increase in the endogenous production of dopamine, without increasing the dosage of BH_4, 1 g BH_4 was, in an additional part of this study, administered in combination with 15 g tyrosine, to 2 patients with Parkinson's disease. Surprisingly, even though a 2-fold increase in the CSF concentration of biopterin was still found, no increase in the CSF concentrations of HVA and 5-HIAA was now observed. It is however known, that tyrosine is an inhibitor of tyrosine hydroxylase (1), and that the activity of tyrosine hydroxylase is reduced in Parkinson's disease (33, 34). The observed events in the CSF may, thus, indicate that the reduced amount of tyrosine hydroxylase in Parkinson's disease is already saturated with its substrate. This would also explain why no major increase in the CSF concentration of HVA was found after the administration of tyrosine alone.

Compared with controls, the patients with Parkinson's disease had, in accordance with earlier findings, reduced concentrations of HVA and 5-HIAA in the CSF (16). The CSF concentration of biopterin was below the normal range in the 2 moderate cases of Parkinson's disease, but normal in the mild and newly diagnosed case. A reduced concentration of biopterin in the CSF probably does reflect a reduced concentration of the BH_4 cofactor. Since a reduced concentration of BH_4, apart from a reduced biosynthesis of dopamine and serotonin, expectedly would produce an increased concentration of phenylalanine, it is noteworthy, that 2 of the patients did indeed have increased concentrations of phenylalanine in the CSF. However, because this material is very small, further studies will be needed to illuminate, whether these results are reproducible.

It has previously been described, that the activity of GTP-cyclohydrolase I (EC 5.5.4.16), is greatly reduced in the Parkinsonian post-

mortem caudate nucleus (35). Reduced activity of GTP-cyclohydrolase I, would expectedly produce a reduced concentration of neopterin in the CSF (Fig. 1). Because the Parkinsonian patients of this study all had a CSF concentration of neopterin within the normal range, we do not however, suspect greatly reduced activity of GTP-cyclohydrolase I.

No side-effects after BH_4 administration have yet been described. The side-effects observed, when patients with Parkinson's disease are treated with L-dopa, even in combination with an inhibitor of peripheral aromatic amino acid decarboxylation, are apparently solely due to increased concentrations of peripheral dopamine (15). Even though BH_4 produced a 4 to 8-fold increase in the CSF concentration of HVA, no increase in the concentration of HVA in the urine was observed. However, treatment with Madopar produced a 13 to 37-fold increase in the urinary excretion of HVA. These observations may be regarded as promising. Thus, if clinical improvement in Parkinson's disease can be produced by treatment with BH_4 in higher dosages, fewer side-effects than with conventional treatment with Madopar may be expected.

Whereas L-dopa treatment of Parkinson's disease is known to cause improvement of principally akinesia and to a lesser extent of rigidity and tremor, no improvement is to be expected in such symptoms of autonomic dysfunction as abnormal heat-regulation, acute sweating attacks, constipation, and oedema in the legs. These symptoms may be improved by treatment with the serotonin precursors L-tryptophan or 5-hydroxytryptophan (15). Furthermore, the depression often following Parkinson's disease has been shown to correlate better with alterations in serotonin metabolites in the CSF than with other neurotransmitters, including dopamine and norepinephrine metabolites (36).

BH_4-administration to patients with Parkinson's disease was in this study shown to produce an increase in the CSF concentration of the serotonin metabolite 5-HIAA. It is thus possible, that further studies may prove BH_4 to be a drug, with a wider range of effects in Parkinson's disease, than any other drug has yet offered.

Acknowledgements

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References

1. Shiman R, Akino M, Kaufman S. Solubilization and partial purification of tyrosine hydroxylase from bovine adrenal medulla. J Biol Chem 1971:*246*:1330–1340.
2. Friedman PA, Kappelman AH, Kaufman S. Partiel purification and characterization of tryptophan hydroxylase from rabbit hindbrain. J Biol Chem 1972:*247*:4165–4173.
3. Kaufman S. The phenylalanine hydroxylating system from mammalian liver. Adv Enzymol 1971:*35*:245–319.
4. Kaufman S, Holtzman NA, Milstien S, Butler IJ, Krumholz A. Phenylketonuria due to a deficiency of dihydropteridine reductase. N Engl J Med 1975:*293*:785–790.
5. Niederwieser A, Staudenmann W, Wang M, Curtius HC, Atares M, Cardesa-Garcia J. Hyperphenylalaninemia with neopterin deficiency - A new enzyme defect presumably of GTP cyclohydrolase. Eur J Pediatr 1982:*138*:97.
6. Rey F, Harpey JP, Leeming RJ, Blair JA, Aicardi J, Rey J. Les hyperphenylalaninemies avec activite normale de la phenylalanine-hydroxylase. Arch Franc Ped 1977:*34*:109–120.
7. Niederwieser A, Ponzone A, Curtius HC. Differential diagnosis of tetrahydrobiopterin deficiency. J Inher Metab Dis 1985:*8* Suppl 1:34–38.
8. McInnes R, Kaufman S, Warsh JJ, Milstien S, Van Loon G, Slyper A, Sherwood G. Neurotransmitter metabolites (NTM) and plasma catachols in biopterin (BP) deficiency. Pediatr Res 1979:*13*:422.
9. Brewster TG, Moskowitz MA, Kaufman S, Breslow JL, Milstien S, Abroms IF. Dihydropteridine reductase deficiency associated with severe neurological disease and mild hyperphenylalaninemia. Pediatr 1979:*63*:94–99.
10. Kaufman S. Hyperphenylalaninaemia caused by defects in biopterin metabolism. J Inher Metab Dis 1985:*8* Suppl 1:20–27.
11. Smith I, Hyland K, Kendall B. Clinical role of pteneridine therapy in tetrahydrobiopterin deficiency. J Inher Metab Dis 1985:*8* Suppl 1:39–45.
12. Ehringer H, Hornykiewicz O. Verteilung von noradralin und dopamin (3-Hydroxytyramin) im Gehirn des Menschen und ihr Verhalten bei Erkrankungen des Extrapyramidalen Systems. Wien klin Wschr 1960:*72*:1236–1239.
13. Birkmayer W, Jellinger K, Riederer P. Striatal and extrastriatal dopaminergic functions. In: Psychobiology of the striatum (Cools, AR ed.) Amsterdam: Elsevier/North-Holland 1977:141–153.
14. Riederer P, Birkmayer W, Seemann D, Wuketich St. Brain-Noradrenaline and 3-Methoxy-4-hydroxyphenylglycol in Parkinson's syndrome. J Neural Transm 1977: *41*:241–251.
15. Birkmayer W, Riederer P.: Parkinson' disease. Biochemistry,
16.
17.
18.
19.
20.
21.
22.
23.
24.
25.
26.

Clinical Pathology and Treatment. Springer Verlag. Wien, New York 1983.

16. Johansson B, Roos BE. 5-Hydroxyindoleacetic and homovanillic acid levels in the cerebrospinal fluid of healthy volunteers and patients with Parkinson's syndrome. Life Sci 1967:*6*:1449-1454.
17. Moir ATB, Ashcroft GW, Crawford TBB, Eccleston D, Guldberg HC. Cerebral metabolites in cerebrospinal fluid as a biochemical approach to the brain. Brain: 1970: *93*:357-368.
18. Guldberg HC, Turner JW, Hanieh A, Ashcroft GW, Crawford TBB, Perry WLM, Gillingham FJ. On the occurrence of homovanillic acid and 5-hydroxyindol-3-ylacetic acid in the ventricular C.S.F. of patients suffering from Parkinsonism. Confin neurol 1967:*29*:73-77.
19. Curzon G, Godwin-Austen B, Tomlinson EB, Kantamaneni BD. The cerebrospinal fluid homovanillic acid concentration in patients with Parkinsonism treated with L-dopa. J Neurol Neurosurg Psyciat 1970:*33*:1-6.
20. Levine RA, Williams AC, Robinson DS, Calne DB, Lovenburg W. Analyses of hydroxylase cofactor activity in the cerebrospinal fluid of patients with Parkinson's Disease. Adv Neurol 1979:*24*:303-307.
21. Nagatsu T, Yamaguchi T, Kato T, Sugimoto T, Matsuura S, Akino M, Nagatsu I, Iizuka R, Narabayashi H. Biopterin in human brain and urine from controls and Parkinsonian patients: Application of a new radioimmunoassay. Clin Chim Acta 1981:*109*:305-311.
22. Blair JA, Parveen H, Barford PA, Leeming RJ. Aetiology of Parkinson's disease (letter) Lancet 1984:*I*:167.
23. Niederwieser A, Staudenmann W, Wetzel E. High-performance liquid chromatography with column switching for the analysis of biogenic amine metabolites and pterins. J Chromatogr 1984:*290*:237-246.
24. Lykkelund C, Nielsen JB, Lou HC, Rasmussen V, Gerdes AM, Christensen E, Güttler F. Increased neurotransmitter biosynthesis in phenylketonuria induced by phenylalanine restriction or by supplementation of unrestricted diet with large amounts of tyrosine. Eur J Pediatr: Accepted for publication.
25. Narabayashi H, Kondo T, Nagatsu T, Sugimoto T, Matsuura S. Tetrahydrobiopterin administration for Parkinsonian symptoms. Proc Japan Acad 1982:*58*; Ser B:283-287.
26. Curtius HC, Niederwieser A, Levine R, Müldner H. Therapeutic efficacy of tetrahydrobiopterin in Parkinson's disease. Adv Neurol 1984:*40*:463-466.
27. Moore AP, Behan PO, Jacobsen W, Armarego WLF. Biopterin in Parkinson's disease. J Neurol Neurosurg Psychiat 1987:*50*:85-87.
28. LeWitt PA, Miller LP, Newman RP, Burns RS, Insel T, Levine RA, Lovenberg W, Calne DB. Tyrosine hydroxylase cofactor (tetrahydrobiopterin) in Parkinsonism. Adv Neurol 1987:*40*:459-462.
29. LeWitt PA, Newman RP, Miller LP, Lovenberg W, Eldridge R. Treatment of dystonia with tetrahydrobiopterin. N Eng J Med 1983:*308*:157-158.
30. Lou HC, Güttler F, Lykkelund C, Bruhn P, Niederwieser A. Decreased vigilance and neurotransmitter synthesis after discontinuation of dietary treatment for phenylketonuria in adolescents. Eur J Pediatr 1985:*144*:17-20.
31. Lou HC, Lykkelund C, Gerdes AM, Udesen H, Bruhn P. Increased Vigilance and dopamine synthesis by large doses of tyrosine or phenylalanine restriction in Phenylketonuria. Acta Pædiatr Scand 1987:*76*:560-565.
32. Wurtman RJ, Larin F, Mostafapour S, Fernstrom JD. Brain catachol synthesis: Control by tyrosine brain concentration. Sci 1974:*185*:183-184.
33. Lloyd KG, Davidson L, Hornykiewicz O. The neurochemistry of Parkinson's disease: Effect of L-dopa therapy. J Pharmacol Exp Ther 1975:*195*:453-464.
34. Nagatsu T, Kato T, Numata (Sudo) Y, Ikuta K, Sano M, Nagatsu I, Kondo Y, Inagaki S, Iizuka R, Hori A, Narabayashi H. Phenylethanolamine N-methyltransferase and other enzymes of catacholamine metabolism in human brain. Clin Chim Acta 1977:*75*:221-232.
35. Nagatsu T, Horikoshi T, Sawada M, Nagatsu I, Kondo T, Iizuka R, Narabayashi H. Biosynthesis of Tetrahydrobiopterin in Parkinsonian human brain. Adv Neurol 1986:*45*:223-226.
36. Mayeux R, Stern Y, Cote L. Altered serotonin metabolism in depressed patients with Parkinson's disease. Neurol 1984:*34*:642-646.

Address
Inger Christine Dissing
Norasvej 30
DK-2920 Charlottenlund
Denmark

The general concept of molecular chaperones

R. JOHN ELLIS

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K.

SUMMARY

This introductory article proposes a conceptual framework in which to consider the information that is emerging about the proteins called molecular chaperones, and suggests some definitions that may be useful in this new field of biochemistry. Molecular chaperones are currently defined in functional terms as a class of unrelated families of protein that assist the correct non-covalent assembly of other polypeptide-containing structures *in vivo*, but which are not components of these assembled structures when they are performing their normal biological functions. The term assembly in this definition embraces not only the folding of newly synthesized polypeptides and any association into oligomers that may occur, but also includes any changes in the degree of either folding or association that may take place when proteins carry out their functions, are transported across membranes, or are repaired or destroyed after stresses such as heat shock. Known molecular chaperones do not convey steric information essential for correct assembly, but appear to act by binding to interactive protein surfaces that are transiently exposed during various cellular processes; this binding inhibits incorrect interactions that may otherwise produce non-functional structures. Thus the concept of molecular chaperones does not contradict the principle of protein self-assembly, but qualifies it by suggesting that *in vivo* self-assembly requires assistance by other protein molecules.

1. HISTORY OF THE MOLECULAR CHAPERONE CONCEPT

The term 'molecular chaperone' was used first to describe the properties of a nuclear protein, nucleoplasmin, in mediating the *in vitro* assembly of nucleosomes from isolated histones and DNA (Laskey *et al.* 1978). This term was chosen because nucleoplasmin mediates nucleosome assembly by preventing incorrect interactions between histones and DNA, without either providing steric information essential for assembly or being a component of the assembled nucleosomes themselves. In these respects, nucleoplasmin is a molecular analogue of the human chaperone, whose traditional role is to prevent incorrect interactions between pairs of human beings, without either providing the steric information necessary for their correct interaction or being present during their married life.

The author came across this term while searching for a precedent for the observation that the assembly of the enzyme rubisco (ribulose bisphosphate carboxylase-oxygenase) in chloroplasts isolated from higher plants also seems to require the transient assistance of another protein that is not a component of the assembled enzyme. The essential finding is that rubisco large subunits, newly synthesized by isolated intact chloroplasts, are bound non-covalently to another abundant protein before transfer to the holoenzyme; it was proposed that this binding might be an obligatory step in rubisco assembly (Barraclough & Ellis 1980). This interpretation did not meet with much enthusiasm at the time because of the general acceptance of the notion of protein self-assembly, which proposes that both the folding and association of polypeptides is a spontaneous process requiring no molecules other than the components of the assembled protein (Caspar & Klug, 1962; Anfinsen 1973).

The suggestion that the rubisco large subunit-binding protein could be regarded as a second example of a molecular chaperone was made at a Royal Society Discussion Meeting on rubisco (Musgrove & Ellis 1986). At this time it was felt that histones–DNA and rubisco subunits might be special cases, as the tendency of both to form non-specific aggregates *in vitro* is so great. However, a speculative paper by Pelham (1986) suggested that the need for a molecular chaperone function may be more widespread. Although he did not use the term 'molecular chaperone', Pelham proposed that members of the heat shock protein 70 (hsp 70) family in animal and microbial cells are involved in the assembly and disassembly of proteins in the nucleus, cytosol and endoplasmic reticulum. Some members of the hsp 70 family are present in unstressed cells, and can bind to denatured or abnormal proteins in a manner reversible by adenosine triphosphate (ATP). Pelham suggested that these proteins have a role in normal protein folding and association in unstressed cells, and are required in increased amounts when proteins have been damaged by stress, both to unscramble protein aggregates which could then refold correctly, and to prevent further damage by binding to exposed hydrophobic surfaces. This seminal paper emboldened the author to propose that all cells contain a variety of proteins that act as molecular chaperones in a number

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of basic cellular processes, such proteins including among others nucleoplasmin, the rubisco large subunit-binding protein and the hsp 70 family (Ellis 1987). This more general proposal has since been steadily extended to a growing range of proteins and cellular processes (Ellis & Hemmingsen 1989; Ellis *et al.* 1989; Rothman 1989; Ellis 1990*a*,*b*; Ellis & van der Vies 1991; Gething & Sambrook 1992; Lorimer 1992; Hartl *et al.* 1992).

One particular family of molecular chaperones are termed the chaperonins after the discovery that the rubisco large subunit-binding protein of chloroplasts is about 50% identical in aminoacyl sequence to the groEL protein of *Escherichia coli* (Hemmingsen *et al.* 1988). The chaperonins are now regarded as just one family within the wider class of molecular chaperones. The specific function of this particular family is to assist the folding of polypeptides in all types of cell; this family should not be confused with other families of molecular chaperone such as the one containing heat shock 70 proteins, which have different functions and aminacyl sequences to the chaperonins. More recent information about some of the different types of molecular chaperone is contained in the following articles in this symposium, whereas this article discusses the general concept.

2. THE MOLECULAR CHAPERONE CONCEPT TODAY

The discovery of molecular chaperones is stimulating a re-examination of a biological process that was thought to be understood: protein assembly. The conventional view of protein assembly, as found in textbooks, is that it is predominantly a process of self-assembly. According to this view, all the information required to specify the structure and function of a protein resides within the aminoacyl sequences of the polypeptides comprising that protein. Interpreted strictly, this view implies that a newly synthesized polypeptide should be able to attain its functional conformation within the intracellular environment with no assistance from other molecules and with no further expenditure of energy. This conformation often produces the ability to associate specifically with other macromolecules, especially other proteins or nucleic acids. This self-assembly principle stems from the classic observations of Fraenkel-Conrat & Williams (1955), who were able to reassemble infectious tobacco mosaic virus by incubating together the separated purified virion components, and by Anson (1945) and Anfinsen (1973), who found that some purified denatured proteins regain their characteristic biological activities on removal of the denaturing agent in the absence of other macromolecules. Although it was speculated that other molecules may assist protein folding *in vivo* (Epstein *et al.* 1963), subsequent generations of researchers studying protein renaturation *in vitro* did not pursue this possibility until the chaperonin family of molecular chaperones was identified by Hemmingsen *et al.* (1988).

Creighton (1984) has pointed out that all the evidence for self-assembly comes from *in vitro* experiments, and the fact that in many cases the denaturation of proteins is not fully reversible *in vitro*, especially at physiological temperatures and at protein concentrations approaching those found *in vivo*, has not until recently raised serious doubt about the validity of the self-assembly principle to describe the *in vivo* situation. The molecular chaperone concept challenges the conventional view by proposing that self-assembly is not the predominant process by which proteins assemble *in vivo*. This proposal is supported by the growing number of instances where proteins will not assemble correctly at the rates and yields required *in vivo* unless other pre-existing proteins are present to assist them. It is this latter group of proteins that are called molecular chaperones.

(*a*) *Definition of the term 'molecular chaperone'*

Molecular chaperones are defined as a functional class of unrelated families of protein that assist the correct non-covalent assembly of other polypeptide-containing structures *in vivo*, but are not components of these assembled structures when they are performing their normal biological functions.

The words used in this definition have been chosen with some care so as not to conflict with future likely discoveries about molecular chaperones or to overlap with existing terms. The definition is based on the function of each molecular chaperone and not on its structure, but it contains no constraints as to the mechanism of that function, hence the use of the imprecise word 'assist'. Thus different molecular chaperones may act in either a catalytic or a non-catalytic manner, may either accelerate or slow down assembly processes, and may either convey steric information essential for assembly or simply inhibit incorrect side-reactions during self-assembly.

All the molecular chaperones studied to date appear to act not by providing steric information essential for assembly but by inhibiting incorrect interactions which produce non-functional structures during self-assembly processes. However, the above definition allows for other mechanisms of action that may be discovered; the *pro*-sequences of subtilisin and α-lytic protease act as molecular chaperones (Silen & Agard 1989) and it is possible, but not established, that they convey steric information essential for the folding of the mature proteases. Thus, by the definition suggested above, only two criteria must be met for a protein to be described as a molecular chaperone: it must in some sense be required for the correct assembly of other protein-containing structures *in vivo* (the mechanism by which it does this being irrelevant), and it must not be a component of the functional assembled structures.

The term 'non-covalent' used in the definition of molecular chaperones is intended to exclude those proteins which perform covalent post-translational modifications to some proteins. Such proteins are often important for protein assembly but are not the molecules under consideration here. Thus, by this definition, protein disulphide isomerase is not a molecular chaperone.

The definition of molecular chaperone proposed above does not require that the molecule possessing the chaperone activity is necessarily synthesized as a separate entity from its target polypeptide-containing structures, only that it is not a component of these structures when they are performing their biological functions. Thus cotranslational (or intramolecular) molecular chaperones, as represented by the *pro*-sequences of some proteases and the ubiquitin tails of some ribosomal precursor proteins (see Ellis & van der Vies 1991), are not excluded by this definition.

(*b*) *Definition of the term 'protein assembly'*

The term 'protein assembly' is used in the context of molecular chaperones in a broad sense; it embraces not only the folding of newly synthesized polypeptide chains and any association into oligomers that may occur, but also any changes in the degree of either folding or association that may take place as proteins perform their normal functions, are transported across membranes, or are repaired or destroyed after damage by stress.

It is important to appreciate this broad use of the term, because some authors use 'protein assembly' in a much more limited sense, to mean just the association of monomers into oligomers. This broad use of the term 'protein assembly' covers the observation that molecular chaperones function not only during several cellular processes under normal conditions, but also to limit damage to proteins caused by stresses such as heat shock. In other words, at least some heat shock proteins function as molecular chaperones, but not all molecular chaperones are heat shock proteins. It is possible to view the stress response as an amplification of a pre-existing molecular chaperone function which all cells require under normal growth conditions, rather than as a novel function induced by stress.

3. WHY DO MOLECULAR CHAPERONES EXIST?

As the principle of protein self-assembly is well supported by *in vitro* studies of many proteins, it is important to ask why this principle should apparently be insufficient in the more complex cellular context. A possible explanation stems from the observation that several fundamental cellular processes involve the transient exposure of interactive protein surfaces to the intracellular environment, and thereby run the risk that these surfaces may interact incorrectly.

The term 'interactive surfaces' refers to any regions of inter- or intra-molecular contact which are important in maintaining protein-containing structures in their biologically active forms. Such surfaces may be either charged or hydrophobic regions, for example, and they may occur on either partly folded or fully folded polypeptides. Cellular processes involving the transient exposure of such surfaces include protein synthesis, protein transport, protein turnover, the disassembly of oligomers (e.g. DNA replication and clathrin cage recycling), the assembly of oligomers inside organelles from monomers either imported or made in *organello*, and protein damage due to environmental stress. As an example, let us consider protein synthesis. This vectorial process produces the aminoterminal region of each polypeptide before the carboxy terminal region. Suppose that the normal fate of the aminoterminal region is to interact with the carboxyterminal region in maintaining the functional structure; this is the case, for example, in cytochrome *c*. We can ask what happens to the aminoterminal region before the carboxyterminal region is made. Can it interact incorrectly with itself or with other molecules in the cell, and, if so, does this present a problem that the cell must combat? Similar questions can be raised about the other processes listed above: in each case, interactive surfaces that at one time are holding protein structures in their active conformations are at another time exposed to the intracellular environment containing high concentrations of many other interactive surfaces.

The self-assembly principle, if applied strictly, implies that all the interactions that take place when such protein surfaces are exposed are totally correct; this means that they are both necessary and sufficient to produce the normal functional conformation. The molecular chaperone concept challenges this view by proposing that in any given assembly process there is a certain probability that incorrect interactions will occur.

(*a*) *Definition of 'incorrect interactions'*

Incorrect interactions are defined as those that give rise to structures which are non-functional in their normal context, i.e. they do not possess the required biological activity.

The probability of incorrect interactions may be so low in some cases that molecular chaperones are not required, but in other cases, e.g. the assembly of nucleosomes and rubisco, the probability of incorrect interactions is so high that molecular chaperones are essential to produce enough functional structures for cellular needs.

According to this view, molecular chaperones are required because many cellular processes involving proteins carry an inherent risk of malfunction. They carry this risk because of the sheer number, variety and flexibility of the many weak interactions that hold proteins in their functional conformations. The cell thus continually faces the problem that incorrect interactions will produce non-functional structures. These ideas can be simplified into a unifying concept by supposing that all cells require a molecular chaperone function.

(*b*) *Definition of the molecular chaperone function*

The molecular chaperone function is defined as the prevention of incorrect interactions between transiently exposed surfaces by the binding of chaperone molecules to those surfaces.

4. MECHANISM OF ACTION OF MOLECULAR CHAPERONES

Present knowledge about the mechanism of action of molecular chaperones (derived principally from studies on nucleoplasmin, the chaperonins and the hsp 70 family) suggests that they function by inhibiting unproductive incorrect assembly pathways which would otherwise act as kinetic dead-end traps. This inhibition is exercised by the non-covalent binding of the molecular chaperone to exposed interactive surfaces to produce stable complexes. Some molecular chaperones, e.g. the chaperonins and the hsp 70 family, can bind to a wide variety of unassembled proteins that are unrelated in sequence, so an important aim of current research is to determine how interactive surfaces are recognized. In these complexes the bound ligands are prevented from interacting incorrectly. Reversal of binding then occurs under circumstances which favour correct interactions involving the ligand. In the case of the chaperonin family, these circumstances include the release of the bound ligand into cages formed at each end of the large oligomeric chaperonin molecules; each cage provides a sequestered environment in which a released polypeptide can fold according to the principle of self-assembly without running the risk of interacting incorrectly with other folding molecules (Saibil *et al.* 1993). In some cases (e.g. the chaperonins and the hsp 70 family), but not all (e.g. nucleoplasmin), this reversal of binding requires ATP hydrolysis. This requirement for energy is another feature by which the molecular chaperone view of protein assembly differs from the conventional view, because it suggests that energy in the form of ATP is often required to assemble proteins as well as to synthesize the peptide bonds.

This model proposed for the action of molecular chaperones suggests that their action is rather subtle, and can be described as assisting self-assembly. Thus the principle of self-assembly is not violated by the molecular chaperone concept, rather it is qualified by the proposal that in the *in vivo* situation self-assembly needs assistance from other protein molecules. On this basis we can distinguish two types of self-assembly.

1. Strict self-assembly: no macromolecules other than the primary structure are required for the polypeptide to have a high probability of assembling correctly within the intracellular environment. (This definition is an over-simplification in that it ignores the post-translational modifications required for some proteins to assemble correctly.)

2. Assisted self-assembly: an appropriate molecular chaperone is required in addition to the primary structure to allow correct assembly to predominate over incorrect assembly; such molecular chaperones convey no steric information over and above that in the primary structure of the ligand.

The ratio of strict self-assembly to assisted self-assembly *in vivo* is not known, but is likely to vary with the spectrum of protein assembly occurring at a given time. It may be that all protein assembly *in vivo* is assisted, because even proteins which self-assemble *in vitro* very rapidly to the active conformation with high yield after removal of denaturant (e.g. dihydrofolate reductase) will bind to a molecular chaperone if presented with one. Perhaps in such cases there is a potential problem of incorrect interactions *in vivo* which has been overlooked by studying the pure protein *in vitro*. The study of protein assembly *in vitro* using pure defined components has enormous analytical advantages, but it also suffers from the limitation that additional components involved *in vivo* may be lost during purification. There is thus a need to repeat all the extensive studies of protein renaturation *in vitro* in the presence of appropriate molecular chaperones.

5. CURRENT LIST OF MOLECULAR CHAPERONES

Table 1 contains a list of proteins that can be regarded as molecular chaperones. They are grouped into families on the basis of aminoacyl sequence, so that

Table 1. *List of molecular chaperones*

(Proteins suggested to be molecular chaperones are grouped on the basis of aminoacyl sequence, together with the cellular functions they are believed to assist. Note that some groups assist the assembly of many different proteins, whereas others are specific for one or a few proteins. The question marks indicate where no firm evidence is available. Hsp = heat shock protein.)

name	proposed roles
nucleoplasmins	nucleosome assembly and disassembly ribosome and ribonucleoprotein particle assembly? transcription?
chaperonins (includes hsp 60 and TCP1)	polypeptide folding polypeptide transport
hsp 70 (or DnaK in *E. coli*)	polypeptide folding polypeptide transport oligomer disassembly
hsp 90	masking of binding sites polypeptide folding?
DnaJ protein	interaction with hsp 70 and GrpE
GrpE protein	interaction with hsp 70
SecB protein	bacterial polypeptide transport
signal recognition particle	polypeptide transport
Pro-sequences	protease folding
ubiquitin tails of some ribosomal proteins	ribosome assembly in eukaryotes
PapD protein	bacterial pilus assembly
PrtM and PrsA	folding of secreted bacterial proteins
Lim protein	folding of bacterial lipase
Rb protein	binding of transcription factors
prions	rogue molecular chaperones?

members within each family are related to one another by sequence but not to members of another family. A theme emerging from recent research is that families of different molecular chaperones cooperate together in defined pathways to assist the assembly of some proteins (Langer *et al.* 1992). This list is predicted to grow as more researchers include the concept of molecular chaperones in their experimental programmes. The following articles discuss recent information about some of these fascinating protein molecules.

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REFERENCES

Anfinsen, C.B. 1973 Principles that govern the folding of protein chains. *Science, Wash.* **181**, 223–230.

Anson, M.L. 1945 Protein denaturation and the properties of protein groups. *Adv. Protein Chem.* **2**, 361–386.

Barraclough, R. & Ellis, R.J. 1980 Protein synthesis in chloroplasts. IX. Assembly of newly-synthesized large subunits into ribulose bisphosphate carboxylase in isolated intact chloroplasts. *Biochim. biophys. Acta* **608**, 19–31.

Caspar, D.L.D. & Klug, A. 1962 Physical principles in the construction of regular viruses. *Cold Spring Harb. Symp. quant. Biol.* **27**, 1–24.

Creighton, T.E. 1984 *The proteins*. New York: W. H. Freeman.

Ellis, R.J. 1987 Proteins as molecular chaperones. *Nature, Lond.* **328**, 378–379.

Ellis, R.J. 1990*a* Molecular chaperones: the plant connection. *Science, Wash.* **250**, 954–959.

Ellis, R.J. (ed.) 1990*b* *Seminars in cell biology*, vol. 1. (71 pages.) Philadelphia: Saunders Scientific.

Ellis, R.J. & Hemmingsen, S.M. 1989 Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. *Trends biochem. Sci.* **14**, 339–342.

Ellis, R.J. & van der Vies, S.M. 1991 Molecular chaperones. *A. Rev. Biochem.* **60**, 321–347.

Ellis, R.J., van der Vies, S.M. & Hemmingsen, S.M. 1989 The molecular chaperone concept. *Biochem. Soc. Symp.* **55**, 145–153.

Epstein, C.J., Goldberger, R.F. & Anfinsen, C.B. 1963 The genetic control of tertiary protein structure: studies with model systems. *Cold Spring Harb. Symp. quant. Biol.* **28**, 439–446.

Fraenkel-Conrat, H. & Williams, R.C. 1955 Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components. *Proc. natn. Acad. Sci. U.S.A.* **41**, 690–698.

Gething, M.-J. & Sambrook, J. 1992 Protein folding in the cell. *Nature, Lond.* **355**, 33–45.

Hartl, F.U., Martin, J. & Neupert, W. 1992 Protein folding in the cell: the role of molecular chaperones hsp 70 and hsp 60. *A. Rev. biophys. biomol. Struct.* **21**, 293–322.

Hemmingsen, S.M., Woolford, C., van der Vies, S.M., Tilly, K., Dennis, D.T., Georgopoulos, C., Hendrix, R.W. & Ellis, R.J. 1988 Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature, Lond.* **333**, 330–334.

Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K. & Hartl, F.U. 1992 Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature, Lond.* **356**, 683–689.

Laskey, R.A., Honda, B.M., Mills, A.D. & Finch, J.T. 1978 Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature, Lond.* **275**, 416–420.

Lorimer, G. 1992 Role of accessory proteins in protein folding. *Curr. Opin. struct. Biol.* **2**, 26–34.

Musgrove, J.E. & Ellis, R.J. 1986 The rubisco large subunit binding protein. *Phil. Trans. R. Soc. Lond.* B **313**, 419–428.

Pelham, H.R.B. 1986 Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell **46**, 956–961.

Rothman, J.E. 1989 Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell* **59**, 591–601.

Saibil, H., Zheng, D., Roseman, A.M., Hunter, A.S., Watson, G.M.F., Chen, S., auf der Mauer, A., O'Hara, B.P., Wood, S.P., Mann, N.H., Barnett, L.K. & Ellis, R.J. 1993 Large ATP-induced conformational changes in a cage-like chaperonin structure. (Submitted.)

Silen, J.L. & Agard, D.A. (1989) The α-lytic protease pro-region does not require a physical linkage to activate the protease domain *in vivo*. *Nature, Lond.* **341**, 462–464.

EXHIBIT H

J. Inherit. Metab. Dis. 24 (2001) 213–230

A structural hypothesis for BH_4 responsiveness in patients with mild forms of hyperphenylalaninaemia and phenylketonuria

H. ERLANDSEN* and R. C. STEVENS*
The Scripps Research Institute, Department of Molecular Biology and Institute for Childhood and Neglected Diseases, La Jolla, CA 92037, USA
**Correspondence: E-mail: stevens@scripps.edu, erlandsen@scripps.edu*

Summary: Deficiencies in the human enzyme phenylalanine hydroxylase (PAH) due to mutations in the PAH gene (*PAH*) result in the inborn error of metabolism phenylketonuria (PKU). The clinical symptom of this disease is an elevated concentration of L-phenylalanine (L-Phe) in blood serum. To prevent mental retardation due to the buildup of neurotoxic metabolites of L-Phe, patients with severe PKU must be treated with a low-L-Phe diet starting early in their life. Owing to extensive newborn screening programmes and genotyping efforts, more than 400 different mutations have been identified in the *PAH* gene. Recently, there have been several reports of PKU patients showing a normalization of their L-Phe concentrations upon oral administration of the natural cofactor to PAH, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4). In an attempt to correlate the clinical responsiveness to BH_4 administration with PKU genotype, we propose specific structural consequences for this subset of PAH mutations. Based on the location and proximity of this subset of mutations to the cofactor-binding site in the three-dimensional structure of PAH, a hypothesis for BH_4 responsiveness in PKU patients is presented. It is believed that some of these mutations result in expressed mutant enzymes that are K_m variants (with a lower binding affinity for BH_4) of the standard PAH enzyme phenotype. Oral administration of excess BH_4 thus makes it possible for these mutant enzymes to suppress their low binding affinity for BH_4, enabling this subset of *PAH* mutations to perform the L-Phe hydroxylation reaction. Most of the BH_4-responsive *PAH* mutations map to the catalytic domain of PAH in either of two categories. Residues are located in cofactor-binding regions or in regions that interact with the secondary structural elements involved in cofactor binding. Based on the series of known mutations that have been found to be responsive to BH_4, we propose that other subsets of *PAH* mutations will have a high likelihood of being responsive to oral BH_4 administration.

The human autosomal recessive metabolic disorder phenylketonuria (PKU; McKusick 261600) and the more benign form of this disorder found in hyperphenylalaninaemia (HPA) are both characterized by elevated concentrations of phenylalanine (L-Phe) and related neurotoxic metabolities in body fluids. This accumulation is most often due to impaired function of the enzyme phenylalanine hydroxylase (PAH; EC 1.14.16.1), caused by mutations in the gene that encodes for PAH (*PAH*; Genbank cDNA Reference Sequence U49897). (The mutations have been catalogued in a curated database which is accessible at the PAH Mutation Analysis Consortium database and web site, located at http://www.mcgill.ca/pahdb (Scriver et al 2000)). Severe PKU (classical PKU) in untreated patients leads to irreversible mental impairment of cognitive development. The current treatment for the majority of clinical phenotypes, ranging from the mild form of non-PKU HPA (characterized by blood L-Phe concentrations of 120–1000 μmol/L) to severe PKU (blood L-Phe >1000 μmol/L), is lifelong dietary control of L-Phe intake.

Owing to the difficulty with compliance to a lifelong low-L-Phe diet, a number of different approaches have been developed in the last few years towards an alternative treatment for PKU. In one approach, a recombinant form of the enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) has been used in an ancillary fashion to degrade phenylalanine into *trans*-cinnamic acid. When PAL was administered orally (in combination with protease inhibitors to inhibit PAL degradation) to mice that were engineered with a model for PKU, this regimen was shown to reduce L-Phe concentrations by up to 50% in only 1 h after administration (Sarkissian et al 1999). The mouse model was generated by *N*-ethyl-*N'*-nitrosourea (ENU) mutagenesis at the *PAH* locus (Sarkissian et al 2000), which resulted in strains of mice that displayed mild, moderate and severe PKU phenotypes relative to control mice. In a second approach, relatively successful gene therapy has been demonstrated in the mouse model, where adenovirus harbouring human *PAH* cDNA was infused into mice (Nagasaki et al 1999). This procedure resulted in measurable expression of PAH in mouse livers. In the case of gene therapy, immunosuppression was necessary to block the host immune response, in order to prolong the duration of *PAH* gene expression for up to 35 days. However, both approaches are still under development and are currently not available for clinical use. With this in mind, it is of interest that Kure and co-workers (Kure et al 1999) recently reported four patients with HPA that showed reduced blood serum L-Phe concentrations in response to oral loading with the natural cofactor BH_4. Genotyping showed that these patients had three different pairs of mutations on the *PAH* alleles. This report by Kure, together with several other case studies reported at the VIII International Congress of Inborn Errors of Metabolism, Cambridge, UK (Spaapen et al 2000; Trefz et al 2000), defines a subset of PKU mutations that are responsive to treatment with the cofactor BH_4 . We presume that there will be more BH_4-responsive mutations that will be discovered in the *PAH* gene that are currently not identified.

Successful cloning and recombinant expression of several disease-causing mutations in the human phenylalanine hydroxylase gene in different eukaryotic,

prokaryotic and cell-free systems have proved invaluable for linking the PKU genotype to the phenotype (Waters et al 1998). Out of the 413 entries currently present in the PKU database (see above) there are 124 different entries for disease-causing mutations that have been recombinantly expressed. These 124 entries map onto only 49 different amino acid positions that have been observed to be mutated in the corresponding disease-causing *PAH* alleles. *In vitro* expression analyses using mammalian cells have shown that *PAH* gene mutations can reduce enzyme activity and stability to a varying extent as well as alter the oligomeric state of the PAH that is produced (Gamez et al 2000; Kayaalp et al 1997).

Based on extensive genotyping, it has been found that most patients suffering from PKU are compound heterozygotes, meaning that they have different mutations on the two *PAH* alleles. It was proposed, and also confirmed for some PKU patients, that the resultant expressed enzymes, enzymatic activities and L-Phe dietary tolerances were a combined product based on the two mutant alleles that were present (Benit et al 1999; Knappskog et al 1996). On a molecular level, this can be explained by the theoretical PAH enzyme tetramers that can be formed from mutated allele 1 (M1) and allele 2 (M2). These possibilities consist of the $(M1)_4$ and $(M2)_4$ homotetramers, and the $(M1)_3(M2)_1$, $(M1)_2(M2)_2$ and $(M1)_1(M2)_3$ heterotetramers. For example, if one of the PKU mutations is found to be more structurally severe (M1) than the other (M2), owing to folding defects, instability or lower amounts of expressed M1 enzyme (Gamez et al 2000), then the PAH tetramer population may consist of varying compositions of the above-mentioned heterotetrameric forms, or solely the $(M2)_4$ homotetramer. For the two PKU patients with fully characterized M1 and M2 alleles (i.e. genotypes L333F/E390G and D143G/G272X), each patient showed an intermediate dietary tolerance for L-Phe, as mentioned above (Benit et al 1999; Knappskog et al 1996). For these two double-mutant *PAH* allele combinations, the PAH enzymatic activity obtained upon coexpression of both mutant PAH sequences corresponded to an average value intermediate between the individual enzymatic activities obtained for the two separate homotetrameric mutants. Alternatively, for severe PKU mutations, such as premature termination deletion mutations (leading to a truncated PAH enzyme without an active site), mutations in introns (leading to splicing defects) or insertion/deletion mutations (leading to a frameshift in the expressed protein), there is little or no active enzyme expressed from the severely mutated allele (Knappskog et al 1996; Okano et al 1991a). Thus, PKU patients harbouring an allele with a severe *PAH* mutation would have to rely exclusively upon PAH residual enzymatic activity generated from a less structurally or functionally severe mutation in the second *PAH* allele (Knappskog et al 1996).

The recently solved crystal structures of human (Erlandsen et al 1997a; Fusetti et al 1998) and rat (Kobe et al 1999) PAH have provided the possibility of determining the structural basis of *PAH* mutations that result in deficient L-Phe hydroxylation (Erlandsen and Stevens 1999; Jennings et al 2000). This structural information has helped formulate rules that may aid in predicting the likely effects of unclassified and newly discovered *PAH* mutations (Jennings et al 2000). Furthermore, with this information we can map any BH_4-responsive mutations that have been observed

in different PKU patients onto the structure of PAH, and with the aid of the recent crystal structure determination of PAH complexed with a cofactor analogue (Erlandsen et al 2000), we can formulate a likely explanation of the molecular basis for these BH_4-responsive *PAH* genotypes.

THE THREE-DIMENSIONAL CRYSTAL STRUCTURE OF PHENYLALANINE HYDROXYLASE

Because of numerous failed attempts to crystallize the full-length wild-type version of human PAH, several smaller truncated forms of the protein were created and, with large scale *E. coli* expression (Martinez et al 1995), crystals were successfully obtained (Erlandsen et al 1997b). The first and highest-resolution crystal structure of phenylalanine hydroxylase was solved using a dimeric double truncated form (Δ1–102/Δ428–452) of human PAH, in which the first 102 residues and the last 24 residues of the PAH sequence were removed from the expression construct (Erlandsen et al 1997a). This structure aided in the determination of a larger, tetrameric structure (Δ1–117) of human PAH in which the first 117 residues were removed (Fusetti et al 1998), as well as a dimeric form of the rat enzyme (Δ430–452) in which the last 22 residues were removed (Kobe et al 1999).

By superimposing the catalytic domain residues 143–410 from the crystal structure of Δ1–117 PAH with the same region of the Δ430–452 PAH structure (Erlandsen and Stevens 1999), a complete full-length structure model of phenylalanine hydroxylase was obtained. There are currently 413 different mutations of the *PAH* locus in the PKU database (Scriver et al 2000); out of these mutations, there are 256 missense, 22 nonsense and 27 silent mutations. The *PAH* mutations that map onto the structural gene of PAH can be divided into five categories, dependent on which portion of the protein structure they affect: (1) residues in the active-site region; (2) structural residues (residues that are important for preserving the intact protein three-dimensional structure); (3) residues involved in interdomain interactions within one monomer; (4) residues involved in interactions with the N-terminal regulatory sequence; and (5) residues at the dimer or tetramer interfaces (Erlandsen and Stevens 1999).

Each monomer in the PAH tetramer has three domains (Figures 1 and 2). Residues 1 to 142 comprise the N-terminal regulatory domain (residues 1–142), which also contains a short autoregulatory sequence (ARS) (residues 19–33) (Figure 1). The ARS covers the opening of the PAH catalytic active site and restricts the access of substrate and cofactor into the active site (Figures 2 and 3). The second region of the protein is the 'basket-like' catalytic domain (residues 143–410), which harbours the active-site iron at the bottom of the 'basket'. The iron is bound by three residues (His-285, His-290 and Glu-330) as well as three water molecules (Erlandsen et al 1997a). In the PAH catalytic mechanism, the iron centre is reduced to the active ferrous (Fe^{2+}) form upon binding the natural cofactor BH_4, followed by subsequent binding of O_2 and the L-Phe substrate. The third portion of the protein is the tetramerization domain (residues 411–452), which consists of an 'arm' formed

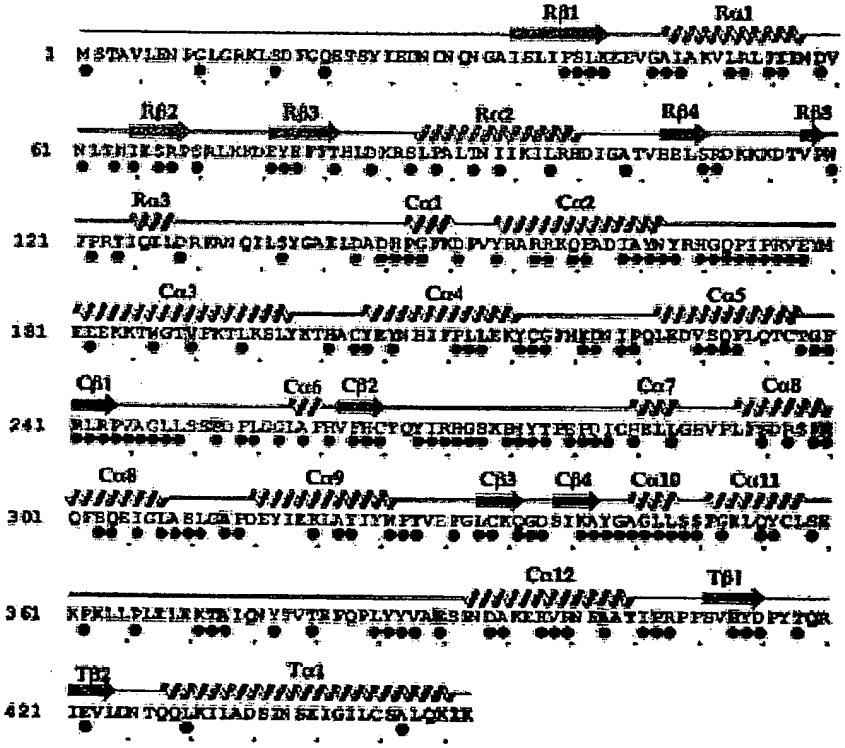


Figure 1 Scheme showing the secondary structure assignment for the human PAH sequence (SWISS-PROT P00439). The secondary structure was assigned using DSSP (Kabsch and Sander 1983) on the composite PAH model (Erlandsen and Stevens 1999). Residues that have PKU mutations associated with them are indicated with red dots. The three residues that are ligands to the active site iron are coloured light blue, and the residues involved in BH_4-responsive phenylketonuria are boxed in green. Secondary structural elements of the regulatory domain are coloured orange, with elements from the catalytic domain coloured grey and elements from the tetramerization domain coloured blue. Residues and secondary structural elements involved in binding of the BH_4 cofactor are coloured purple

by two β-strands and a 40 Å long α-helix. This helix is responsible for forming the intact PAH tetramer, through interdomain shared coiled-coil interactions in the centre of the tetramer with the three other monomers (Figure 2A–C).

STRUCTURE-BASED CHARACTERIZATION OF BH_4-RESPONSIVE PKU MUTATIONS

As previously mentioned, there have been a number of reports in which patients with mild HPA have been able to bring their blood L-Phe concentrations back to normal

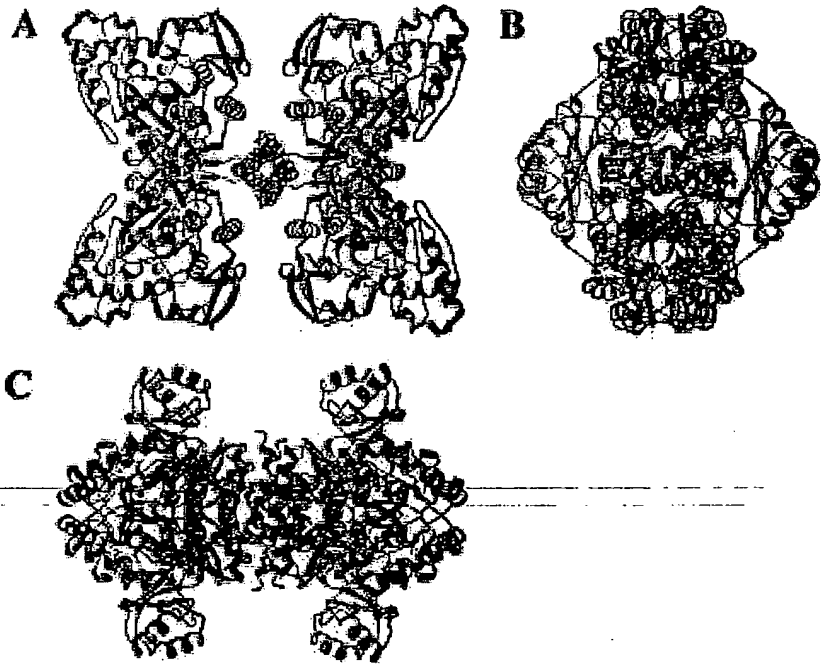


Figure 2 Three views of the composite model of phenylalanine hydroxylase. The regulatory domain (residues 19–142) is coloured orange, the catalytic domain (residues 143–410) is coloured grey, and the tetramerization domain (residues 411–452) is coloured blue. The active-site iron is shown as a yellow sphere. The regions of the backbone involved in cofactor binding are coloured purple. (A) Front view. (B) Side view, seen in the plane of the paper along the *x*-axis as compared to (A). (C) Side view, seen in the plane of the paper along the *y*-axis as compared to (A). The figures were produced with the programs Molscript (Kraulis 1991) and Raster 3D (Merritt and Bacon 1997)

levels by taking BH_4 orally (Kure et al 1999; Spaapen et al 2000; Trefz et al 2000). Known BH_4-responsive heterozygote genotype examples have been compiled in Table 1, together with relevant data on homozygote HPA genotypes. The three-dimensional structural locations of the residues involved in these BH_4-responsive genotypes are all shown in Figure 3. Kure and co-workers (1999) stated the important assumption that if responsiveness towards BH_4 is determined by the mutations present in the *PAH* gene, then patients with PKU who have the same mutations should also respond similarly to BH_4 loading. In addition, Trefz and colleagues (2000) speculated that the biochemical basis for the responsiveness to BH_4 in certain PKU patients was due to the PAH enzyme having a variant K_m, where

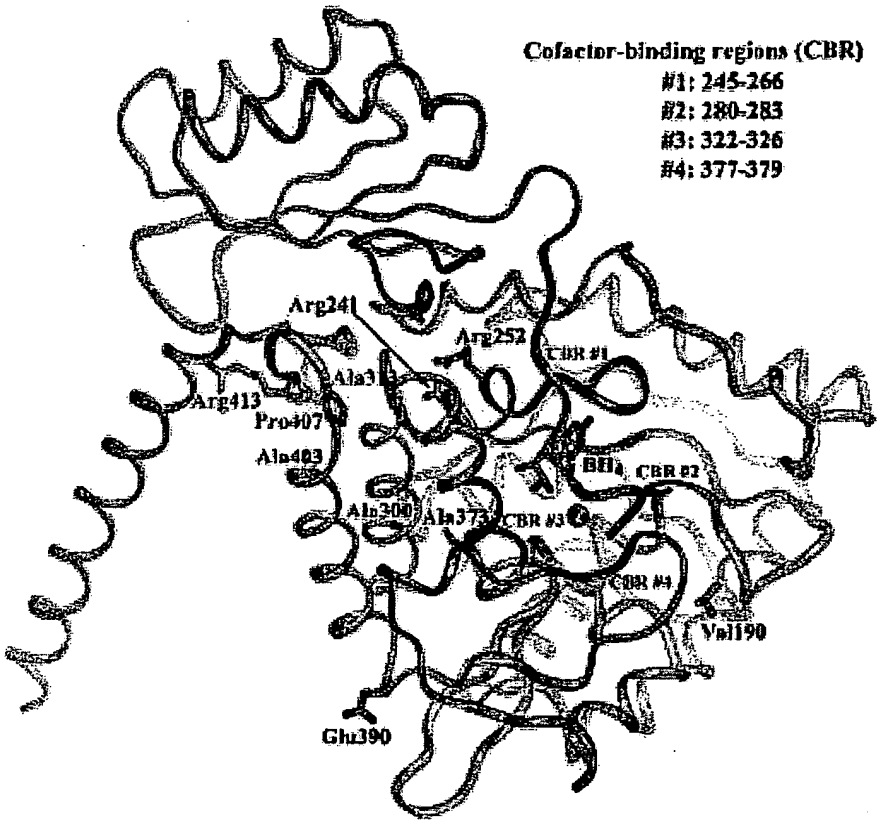
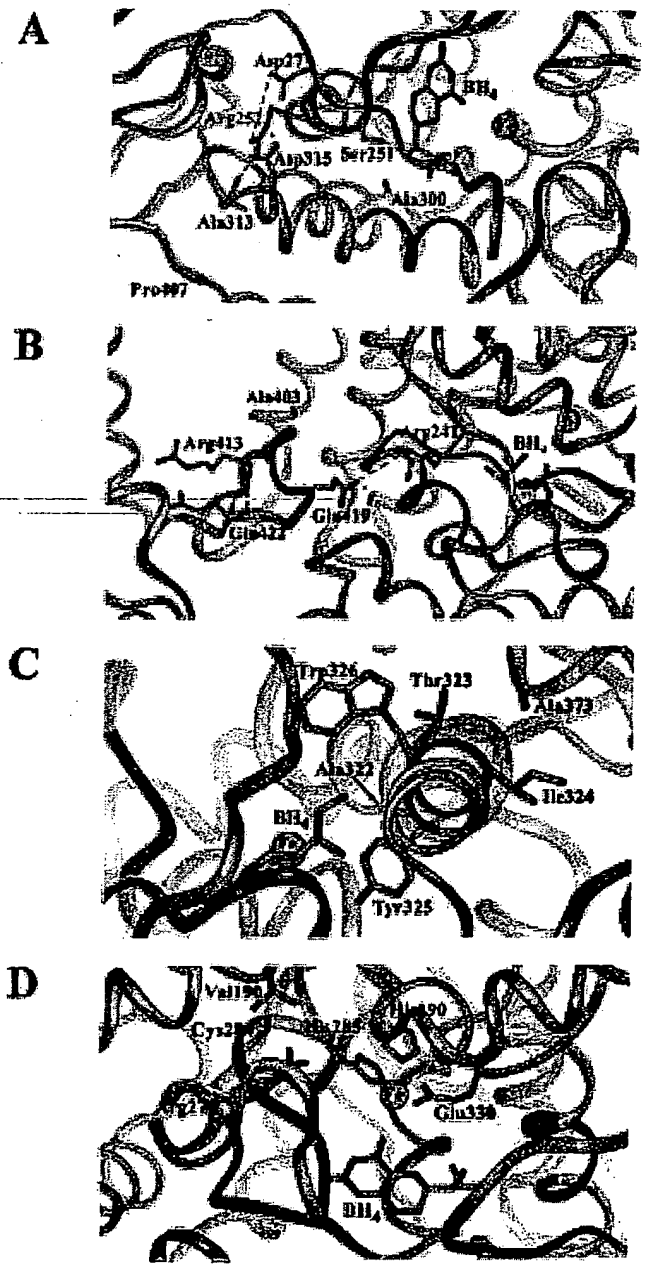


Figure 3 View of the protein backbone for a monomer from the composite model of phenylalanine hydroxylase. The same colour scheme as in Figure 2 is used. Side-chains of the residues involved in PKU patients found to be BH_4-responsive are shown (see Table 1). Side-chain carbon atoms are coloured green, with nitrogen atoms coloured blue and oxygen atoms coloured red. The active-site iron is shown as a yellow sphere. The cofactor BH_4 is positioned into the structure on the basis of the electron density observed for the cofactor analogue 7,8-dihydro-L-biopterin found in the crystal structure of human double-truncated phenylalanine hydroxylase (Erlandsen et al 2000). The cofactor-binding regions (CBR) are shown in purple. These are CBR #1 (residues 245–266), CBR #2 (residues 280–283), CBR #3 (residues 322–326) and CBR #4 (residues 377–379). The figure was produced with the program INSIGHT, which is available from Molecular Simulations Inc.

enhanced residual activity is achieved by oral supplementation of BH_4. Thus, the mutant PAH retains BH_4-binding affinity; however, the reaction occurs at a lower rate compared to wild-type PAH. Structurally, a likely explanation for this effect is that the PAH mutation reduces the cofactor-binding affinity at the active site


A
Asp27
BH4
Arg25
Asp315
Ser251
Ala300
Ala313
Pro307
B
Ala403
Arg413
BH4
Glu419
Glu422
C
Tyr326
Thr323
Ala373
Ala327
Ile324
BH4
Tyr325
D
Val190
Cys285
Glu330
BH4

as a result of slight distortions of the active-site pocket and cofactor-binding site. However, the structural modifications due to PAH mutation are not enough to completely abolish cofactor binding in the BH_4-responsive mutants, and flooding of the active site with cofactor in these situations restores the activity of the enzyme. Consequently, substrate turnover is achieved with BH_4 loading.

Regions that interact with the cofactor analogue in the crystal structure of PAH with 7,8-dihydro-L-biopterin (7,8-BH_2) (Erlandsen et al 2000) are assumed also to interact with the natural BH_4 cofactor. Since BH_4 and 7,8-BH_2 are almost identical (except for two hydrogen atoms in position N-3 and N-5 on the pteridine rings), the same binding site is assumed to exist for both molecules (Figure 3). Thus, the natural cofactor BH_4 was placed into the electron density for 7,8-BH_2. Residues shown to interact with the cofactor analogue in the PAH crystal structure are Gly-247, Leu-249, Ser-251, Phe-254, His-264, Glu-286, Ala-322 and Tyr-325. These residues, which interact with or are close in proximity to the cofactor, have been included in the cofactor-binding regions (CBR). These regions are CBR #1 (residues 245–266), CBR #2 (residues 280–283), CBR #3 (residues 322–326) and CBR #4 (residues 377–379). These four cofactor-binding regions are shown in purple ribbon backbone representations in Figure 3, along with the positions of residues involved with the BH_4-responsive PKU genotypes. The locations of BH_4 and iron in the PAH active site are shown for easier orientation in both Figures 3 and 4.

The first clinical study that reported BH_4 responsiveness in patients with mild HPA (Kure et al 1999) found that the serum L-Phe levels of some patients gradually decreased after an oral loading of BH_4 (corresponding to a dose of 5–10 mg/kg bodyweight). The patients' baseline urinary pteridine and dihydropteridine reductase (the enzyme responsible for natural regeneration of BH_4) (EC 1.6.99.7) levels in these patients were normal, suggesting that these patients were deficient in PAH activity rather than BH_4. Analysis of the *PAH* genotypes for these patients showed the following mutations: P407S/R252W, IVS4−1g>a/A373T, and R413P/R241C. The latter genotype was found in two patients in the same study (Kure et al 1999), and both patients showed the same responsiveness towards BH_4. One patient in the study was found not to respond to BH_4 (genotype P407S/R111X). This patient shares one mutant allele (P407S mutation) with a patient who did

Figure 4 Close-up views of selected residues involved in BH_4 responsiveness along with their interactions. The same colour scheme as in Figures 2 and 3 is used. The active-site iron and the cofactor are also shown. (A) Selected residues involved in the genotypes R252W/P407S, A313T/1099insC and A300S/A403V. (B) Important residues involved in the R413P/R241C and the R241C/A403V genotypes. (C) View of the region around residue Ala-373, which is involved in the BH_4-responsive IVS4−1g>a/A373T genotype, along with additional side-chain locations for residues in close proximity to Ala-373. (D) View of the region around residue Val-190, which is involved in the BH_4-responsive V190A/R243X genotype, together with selected side-chain locations for residues in close proximity to Val-190 and the active-site iron along with the residues acting as ligands to the iron centre. The figures were produced with the program INSIGHT, which is available from Molecular Simulations Inc.

Table 1 Genotype, phenotype and residual enzymatic activities for BH_4-responsive mutations and comparison with homozygotes

PKU genotype					
Allele 1	*Allele 2*	*BH_4-responsive*	*PKU phenotype*	*Residual enzymatic activity*	*Reference*
R252W	R252W	?	Classical PKU	R252W; <1% (COS cells)	Okano et al (1991b)
P407S	R252W	+	Mild HPA	Data not available	Kure et al (1999)
P407S	R111X	−	Mild HPA	Data not available	Kure et al (1999)
IVS4−1g>a	A373T	+	Mild HPA	Data not available	Kure et al (1999)
IVS4−1g>a	IVS4−1g>a	?	Classical PKU	Data not available	Kure et al (1999)
R413P	R241C	+	Mild HPA	R413P; <3% (COS cells) R241C; 25% (COS cells)	Kure et al (1999), Okano et al (1994)
R413P	R413P	?	Classical PKU	R413P; <3% (COS cells)	Wang et al (1991)
IVS10nt−11g>a	E390G	+	Mild HPA	E390G; 70% (COS cells)	Trefz et al (2000)
A313T	1099insC	+	Variant HPA	Data not available	Spaapen et al (2000)
V190A	R243X	+	Variant HPA	R243X; <1% (COS cells)	Okano et al (1991a), Spaapen et al (2000)
A300S	A403V	+	Variant HPA	A403V; 32% (COS cells)	Spaapen et al (2000), PKU database
R241C	A403V	+	Variant HPA	R241C; 25% (COS cells) A403V; 32% (COS cells)	Okano et al (1991b), Spaapen et al (2000), PKU database

respond to BH_4 (Table 1). Table 1 lists two other reports of patients with BH_4-responsive PKU that were found to have several other genotypes: IVS10nt−11g>a/E390G (Trefz et al 2000), A313T/1099insC, V190A/R243X, A300S/A403V, and R241C/A403V (Spaapen et al 2000). In the following discussion, we will present a molecular rationale for each genotype that has been observed to have BH_4 responsiveness.

THE P407S/R252W GENOTYPE

One of the first genotypes found to be responsive to BH_4 was P407S/R252W. Neither residue in this genotype interacts directly with the cofactor, but Arg-252 follows Ser-251 in the PAH sequence and both residues are located in CBR #1 (Figures 1 and 3). Ser-251 is presumed to position BH_4 by hydrogen bonding to the dihydroxypropyl side-chain, placing the BH_4 cofactor in the correct orientation for catalysis to occur (Figure 4A). Arg-252 hydrogen bonds to residue Ala-313 (through the backbone carbonyl oxygen of Ala-313), and also forms hydrogen bonds with Asp-315 and Asp-27 (which is located in the autoregulatory sequence (ARS) that covers the active site). Mutation of Arg-252 into a tryptophan or glycine, as found in the R252G PKU mutation, would remove these stabilizing hydrogen bonds and interfere with the proper interaction between the ARS and the PAH active site (Figure 4A). Residues Ala-313 and Asp-315 are located in a loop (residues 312–315) that forms hydrogen bonds to the first β-strand of the tetramerization domain. Elimination of these stabilizing interactions within the tetramerization domain upon mutating Arg-252 might explain the low yield of oligomeric forms of PAH obtained upon expression of the R252G mutant in *E. coli* (Bjørgo et al 1998). Patients homozygous for the R252W *PAH* mutation display classical PKU, and when PAH containing the R252W mutation is expressed in COS cells, less than 1% of wild-type activity is obtained (Okano et al 1991b). The second allele in the R252W/P407S genotype involves residue Pro-407, which precedes the start of the tetramerization domain. Mutation into a less rigid residue (serine) might inhibit the formation of PAH tetramers; however, the PAH monomers might still retain some cofactor-binding affinity and catalytic activity. Kure and colleagues (1999) reported a non-BH_4-responsive patient with the P407S/R111X genotype that also contains this P407S allele. The R111X mutation results in a truncated PAH enzyme that contains only a partial regulatory domain sequence and presumably does not have any enzymatic activity. Therefore, any PAH enzymatic activity for the P407S/R111X genotype must be due to the P407S mutant enzyme. Based on the difference in phenotype and BH_4 responsiveness for the R252W/P407S and P407S/R111X genotypes, responsiveness to BH_4 is determined not only by the specific *PAH* mutations that are present but also by the molecular composition of the PAH mutant subunits present in the tetramer.

THE R413P/R241C GENOTYPE

The R413P/R241C genotype was also found by Kure and co-workers (1999) to be responsive to cofactor loading. Arg-241 is located in a surface region of the catalytic domain prior to CBR #1 (Figure 4B), at the start of a short β-strand (Cβ1) (Figure 1). In the tetrameric structure (Δ1–117) of PAH (Fusetti et al 1998), Arg-241 was observed to be hydrogen bonded to Gln-419, which is located in the tetramerization domain, and thus position 241 participates in important interdomain interactions between the catalytic domain and the tetramerization domain (Figure 4B). However, Arg-241 is not conserved when compared to the other two homologous enzymes in the tetrameric aromatic amino acid hydroxylase superfamily, tyrosine hydroxylase (EC 1.14.16.2) and tryptophan hydroxylase (EC 1.14.16.4), where position 241 is a glutamine and a serine, respectively. Thus, it cannot be solely responsible for proper tetramer formation. Three PKU *PAH* mutations are associated with Arg-241: R241H, R241L and R241C. When either the R241C or R241H mutated PAH constructs are expressed in COS cells, the expressed enzymes display 25% (Okano et al 1994) and 23% (PKU database) activity, respectively, as compared to wild-type PAH. The second position of mutation in this genotype, Arg-413 is located in the tetramerization domain, and hydrogen bonds to Glu-422. This hydrogen bond is one of a limited number of hydrogen bonds that are responsible for holding together the β-ribbon (formed by Tβ1 and Tβ2) (Figure 1) that is located in the hinge region of the tetramerization domain. Formation of this β-ribbon ensures proper tetramer formation by positioning the 40 Å long α-helix for coiled-coil interactions with other PAH monomers. Three PKU *PAH* mutations have been found for Arg-413: R413S, R413C and R413P. The R413P mutated PAH construct, when expressed in COS cells, results in an enzyme that has <3% (Wang et al 1991) activity when compared to wild-type PAH. Patients homozygous for the R413P mutation have clinical symptoms of classical PKU. Thus, the R241C mutation, owing to its mild effect on the structure (and active site) as compared to Arg-413, is presumably responsible for the observed BH_4 responsiveness in the R413P/R241C genotype. One might predict, based on the known residual enzymatic activity of the R241H mutation (23%; PKU database), that this mutation would give similar BH_4 responsiveness to that observed for R241C, even in combination with a more severe mutation such as R413P.

THE IVS10nt−11g>a/E390G GENOTYPE

Trefz and colleagues (2000) reported on another PKU patient that was found to be responsive to BH_4 oral loading. In the IVS10nt−11g>a/E390G phenotype, the latter mutation must be responsible for the observed BH_4-responsiveness in this genotype, since the first mutation results in a splicing defect in intron 10 of the *PAH* gene, leading to no functional protein being expressed for this allele. A PAH construct containing the Gly-390 mutation shows 70% of wild-type activity when expressed in COS cells (PKU database). In the structure of tetrameric PAH (Fusetti et al 1998), Glu-390 is located on the surface of the protein in the catalytic domain, following

CBR #4 in the primary sequence (Figures 1 and 3). The Glu-390 side-chain is pointing towards the catalytic domain of a second monomer in the tetrameric structure and also points towards the central tetramerization domain. Since Glu-390 is located on the surface, it forms no hydrogen bonds, and mutation into a flexible glycine at this position must be slightly destabilizing for the protein backbone. The Gly-390 mutant PAH, as found for the other BH_4-responsive *PAH* mutations, most likely retains its cofactor binding affinity, possibly with a slight increase in K_m. However, the molecular effect of the E390G mutation is difficult to predict on the basis of the structure. A better hypothesis might be developed by performing kinetic measurements on cofactor binding to the recombinantly expressed mutant enzyme.

THE IVS4−1g>a/A373T GENOTYPE

A final BH_4-responsive genotype reported by Kure and co-workers (1999) was IVS4−1g>a/A373T. The patient with this genotype had a mild HPA phenotype. The IVS4−1g>a mutation affects intron 4 and results in no functional protein being expressed, similarly to the IVS10nt−11g>a mutation in the genotype mentioned previously. Patients who are homozygous for the IVS4−1g>a mutation display clinical symptoms of classical PKU. The second mutation involves position 373. Ala-373 is located in a region that forms hydrophobic interactions with helix Cα9, which contains Tyr-325, Trp-326 and Ala-322 (all in CBR #3) (Figure 4C). The backbone carbonyl oxygen of residue Ala-322 hydrogen bonds to the dihydroxypropyl side-chain of the cofactor. Substitution into a threonine at position 373 might be accommodated relatively easily with respect to the packing in this region of the protein, and might not result in too much structural perturbation in the Cα9 helix or the active site. The minor structural changes caused by the A373T mutation must not be too severe, since this PAH mutant retains binding affinity for BH_4, and provides for the observed BH_4 responsiveness of this *PAH* genotype.

THE A313T/1099insC AND V190A/R243X GENOTYPES

Patients with the genotype A313T/1099insC show clinical symptoms of variant HPA and are responsive to BH_4 loading, as reported by Spaapen et al (2000). The allele responsible for this behaviour is most likely the A313T mutation, since the 1099insC mutation results in a frameshift after Leu-367. However, since this frameshift mutation contains all the residues needed for binding the cofactor, it is not ruled out that the 1099insC mutant PAH might possibly exhibit some enzymatic activity. Unfortunately, no expression information for either of these two mutants is available. The backbone carbonyl oxygen of Ala-313 hydrogen bonds to Arg-252 (in CBR #1) in the tetrameric PAH structure (Figure 4A). Position 313 is located in a loop between helices Cα8 and Cα9, and at the interface to the regulatory domain (close to Pro-119). The residue adjacent to Ala-313 is Gly-312, and this flexible glycine residue might compensate for any structural distortions introduced

by a threonine substitution at position 313. Thus, the A313T substitution might not affect cofactor binding too much.

Similarly, a patient with genotype V190A/R243X also displayed clinical symptoms of variant HPA. Here the responsive mutation must be the V190A mutation, since the R243X mutation results in a truncated enzyme missing all of the residues involved in active-site iron and cofactor binding. Residue Val-190 is located in helix Cα3, in close proximity to the backbone atoms of His-285, and approximately 4 Å away from both the Cβ atom of Cys-284 and the side-chain of Arg-270 (Figure 4D). Substitution into an alanine at position 190 conserves hydrophobicity; however, alanine is a smaller residue than valine. Therefore, substitution into an alanine putatively creates an open space where the two methyl groups of the valine were located. Unless this space is filled with water molecules, the resultant mutant enzyme with Ala-190 might have Cys-284 (which is at the end of CBR #2) and also the backbone of His-285 move into the empty space created by the Ala-190 substitution. This structural rearrangement would alter the cofactor-binding site, potentially lowering the cofactor binding affinity and requiring additional cofactor to be present for substrate turnover, thus explaining the BH_4 responsiveness observed.

THE A300S/A403V AND R241C/A403V GENOTYPES

The two genotypes A300S/A403V and R241C/A403V were both found in variant HPA patients who were observed to be responsive to BH_4 (Spaapen et al 2000). These genotypes share one mutant allele, the A403V allele. Residue Ala-403 is located at the end of helix Cα12, in close proximity to the side-chain of Ala-309 in helix Cα8 (3.5 Å). An alanine or a similar small residue might be necessary at this position for helices Cα8 and Cα12 to pack closely together. Thus, the substitution into a larger valine at this position as found in th A403V mutation might result in a less stable protein being produced. The R241C/A403V genotype shares the R241C allele with the previously mentioned genotype R413P/R241C, where it was concluded that the R241C mutation was responsible for the observed BH_4 responsiveness. This conclusion might therefore be the case for the R241C/A403V genotype as well. In the A300S/A403V genotype, the Ala-300 residue is located in the middle of helix Cα8 (Figure 4A), which is lining the bottom of the active site. Ala-300 does not form any hydrogen bonds, and a substitution into a serine at this position might be accommodated with only minor distortions in the structure. The closest residue to Ala-300 is Arg-261, which is located 4.3 Å away from the side-chain of Ala-300 in CBR #1 (residues 245–266). The substitution into a slightly larger serine at position 300 could cause the Arg-261 side-chain to move, thus changing the shape of the cofactor-binding site. Thus, the Ser-300 substitution would likely lower the binding affinity for BH_4 in the mutant enzyme as compared to the wild-type enzyme, explaining the BH_4 responsiveness of the A300S/A403V genotype.

On the basis of the eight genotypes that have been found to be responsive to BH_4, we can propose generalized rules for allowable mutations that will potentially

respond to BH_4 treatment. First, none of the BH_4-responsive mutations should be residues that are directly interacting with the cofactor. These mutations would lower the binding affinity of the cofactor too much, which would prevent appreciable turnover of L-Phe substrate. Second, the BH_4-responsive mutations can be located in the cofactor-binding regions (CBR #1 (residues 245–266), CBR #2 (residues 280–283), CBR #3 (residues 322–326) and CBR #4 (residues 377–379)), or in regions that closely interact with the cofactor-binding regions. However, none of the single point mutations in the *PAH* gene can be too structurally severe; that is, allowed mutations would not involve proline residues or substitutions from small residues (such as glycine or alanine) into larger residues (such as tryptophan, arginine or lysine). These severe mutations would very likely cause too much structural distortion to the PAH architecture, and it would not be expected that the cofactor-binding site would be conserved in these instances.

CONCLUSIONS

There have been several recent reports of patients with mild HPA to variant HPA, who were found to be responsive to oral loading of BH_4. It is believed that these *PAH* mutations result in mutant enzymes that are K_m variants of the PAH enzyme phenotype (Trefz et al 2000) that are still able to bind the cofactor. Oral addition of excess BH_4 makes it possible for the mutant enzymes to overcome the lowered binding of BH_4 and perform the L-Phe hydroxylation reaction at a lower rate. Thus, L-Phe concentrations return to 'safe' amounts and the patients can, by taking BH_4 supplement, avoid the low-L-Phe diet. When mapped onto the crystal structure of phenylalanine hydroxylase, none of the observed mutations are located at residues that interact with the cofactor. Most of the mutations that lead to BH_4 responsiveness map onto the catalytic domain in two categories. These residues are located either in the cofactor-binding regions (Figure 3) or in regions that interact with the secondary structural elements that are involved in cofactor binding. Thus, it becomes possible to predict (to some extent) which PAH mutant residues that have PKU and HPA phenotypes associated with them, might be treatable with excess BH_4.

Some logical criteria limit the *a priori* prediction of residues that, when mutated, would not be responsive to BH_4. From the known effects of mutations in proteins (Durr and Jelesarov 2000; Ghosh et al 1999; Harris et al 2000; Hubbard and Argos 1995; Oue et al 1999; Zaremba and Gregoret 1999) we can determine a subset of nonresponsive mutations. None of the missense mutations that would be described in the two categories mentioned above should be too 'structurally severe', meaning that the mutations should not involve prolines in structurally important positions or substitutions from a small amino acid (like glycine or alanine) into a larger residue. It is very likely that these gross mutations would cause too severe a structural distortion, causing major folding defects in any expressed protein harbouring these mutant alleles.

In order to provide further evidence to support our BH_4 responsiveness structural hypothesis, it is imperative that an experimental database be developed to facilitate the prediction of *PAH* genotypes that would be treatable with BH_4. This will require

further BH_4 loading tests and patient genotyping. Subsequent expression analyses and kinetic measurements of the recombinantly expressed mutant proteins with the natural cofactor BH_4 will be required to supplement our current understanding of the genotype–phenotype relationship for PKU patients. Unfortunately, very few groups are currently performing these types of analyses, possibly because of the lack of expertise or the presumed lack of necessity for this type of information for actual treatment of PKU patients. However, from the compelling data summarized in this paper, there does seem to be a need to predict BH_4-responsive mutations.

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REFERENCES

Benit P, Rey F, Blandin-Savoja F, Munnich A, Abadie V, Rey J (1999) The mutant genotype is the main determinant of the metabolic phenotype in phenylalanine hydroxylase deficiency. *Mol Genet Metab* **68**: 43–47.

Bjørgo E, Knappskog PM, Martinez A, Stevens RC, Flatmark T (1998) Partial characterization and three-dimensional-structural localization of eight mutations in exon 7 of the human phenylalanine hydroxylase gene associated with phenylketonuria. *Eur J Biochem* **257**: 1–10.

Durr E, Jelesarov I (2000) Thermodynamic analysis of cavity creating mutations in an engineered leucine zipper and energetics of glycerol-induced coiled coil stabilization. *Biochemistry* **39**: 4472–4482.

Erlandsen H, Stevens RC (1999) The structural basis of phenylketonuria. *Mol Genet Metab* **68**: 103–125.

Erlandsen H, Fusetti F, Martinez A, Hough E, Flatmark T, Stevens RC (1997a) Crystal structure of the catalytic domain of human phenylalanine hydroxylase reveals the structural basis for phenylketonuria. *Nature Struct Biol* **4**: 995–1000.

Erlandsen H, Martinez A, Knappskog PM, Haavik J, Hough E, Flatmark T (1997b) Crystallization and preliminary diffraction analysis of a truncated homodimer of human phenylalanine hydroxylase. *FEBS Lett* **406**: 171–174.

Erlandsen H, Bjørgo E, Flatmark T, Stevens RC (2000) Crystal structure and site-specific mutagenesis of pterin-bound human phenylalanine hydroxylase. *Biochemistry* **39**: 2208–2217.

Fusetti F, Erlandsen H, Flatmark T, C. SR (1998) Structure of tetrameric human phenylalanine hydroxylase and its implications for phenylketonuria. *J Biol Chem* **273**: 16962–16967.

Gamez A, Perez B, Ugarte M, Desviat LR (2000) Expression analysis of phenylketonuria mutations. Effect on folding and stability of the phenylalanine hydroxylase protein. *J Biol Chem* **275**: 29737–29742.

Ghosh S, Wolan D, Adak S, et al (1999) Mutational analysis of the tetrahydrobiopterin-binding site in inducible nitric-oxide synthase. *J Biol Chem* **274**: 24100–24112.

Harris TK, Wu G, Massiah MA, Mildvan AS (2000) Mutational, kinetic, and NMR studies of the roles of conserved glutamate residues and of lysine-39 in the mechanism of the MutT pyrophosphohydrolase. *Biochemistry* **39**: 1655–1674.

Hubbard SJ, Argos P (1995) Evidence on close packing and cavities in proteins. *Curr Opin Biotechnol* **6**: 375–381.

Jennings IG, Cotton RG, Kobe B (2000) Structural interpretation of mutations in phenylalanine hydroxylase protein aids in identifying genotype-phenotype correlations in phenylketonuria. *Eur J Hum Genet* **8**: 683–696.

Kabsch W, Sander C (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **22**: 2577–2637.

Kayaalp E, Treacy E, Waters PJ, Byck S, Nowacki P, Scriver CR (1997) Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a metanalysis of genotype-phenotype correlations. *Am J Hum Genet* **61**: 1309–1317.

Knappskog PM, Eiken HG, Martinez A, Bruland O, Apold J, Flatmark T (1996) PKU mutation (D143G) associated with an apparent high residual enzyme activity: expression of a kinetic variant form of phenylalanine hydroxylase in three different systems. *Hum Mutat* **8**: 236–246.

Kobe B, Jennings IG, House CM, et al (1999) Structural basis of autoregulation of phenylalanine hydroxylase. *Nature Struct Biol* **6**: 442–448.

Kraulis PJ (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr* **24**: 946–950.

Kure S, Hou DC, Ohura T, et al (1999) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *J Pediatr* **135**: 375–378.

Martinez A, Knappskog PM, Olafdottir S, et al (1995) Expression of recombinant human phenylalanine hydroxylase as fusion protein in *Escherichia coli* circumvents proteolytic degradation by host cell proteases. *Biochem J* **306**: 589–597.

Merritt EA, Bacon DJ (1997) Raster3D: photorealistic molecular graphics. *Methods Enzymol* **277**: 505–524.

Nagasaki Y, Matsubara Y, Takano H, et al (1999) Reversal of hypopigmentation in phenylketonuria mice by adenovirus-mediated gene transfer. *Pediatr Res* **45**: 465–473.

Okano Y, Eisensmith RC, Guttler F, et al (1991a) Molecular basis of phenotypic heterogeneity in phenylketonuria. *N Engl J Med* **324**: 1232–1238.

Okano Y, Wang T, Eisensmith RC, et al (1991b) Phenylketonuria missense mutations in the Mediterranean. *Genomics* **9**: 96–103.

Okano Y, Hase Y, Shintaku H, et al (1994) Molecular characterization of phenylketonuric mutations in Japanese by analysis of phenylalanine hydroxylase mRNA from lymphoblasts. *Hum Mol Genet* **3**: 659.

Oue S, Okamoto A, Yano T, Kagamiyama H (1999) Redesigning the substrate specificity of an enzyme by cumulative effects of the mutations of non-active site residues. *J Biol Chem* **274**: 2344–2349.

Sarkissian CN, Shao Z, Blain F, et al (1999) A different approach to treatment of phenylketonuria: phenylalanine degradation with recombinant phenylalanine ammonia lyase. *Proc Natl Acad Sci USA* **96**: 2339–2344.

Sarkissian CN, Boulais DM, McDonald JD, Scriver CR (2000) A heteroallelic mutant mouse model: a new orthologue for human hyperphenylalaninemia. *Mol Genet Metab* **69**: 188–194.

Scriver CR, Waters PJ, Sarkissian C, et al (2000) PAHdb: a locus-specific knowledgebase. *Hum Mutat* **15**: 99–104.

Spaapen LJM, Bakker JA, Velter C, et al (2000) Tetrahydrobiopterin-responsive hyperphenylalaninemia (HPA) in dutch neonates. *J Inherit Metab Dis* **23**: (supplement 1): 45.

Trefz F, Blau N, Aulehla-Scholz C, Korall H, Frauendienst-Egger G (2000) Treatment of mild phenylketonuria (PKU) by tetrahydrobiopterin (BH4). *J Inherit Metab Dis* **23** (supplement 1): 47.

Wang T, Okano Y, Eisensmith RC, et al (1991) Founder effect of a prevalent phenylketonuria mutation in the Oriental population. *Proc Natl Acad Sci USA* **88**: 2146–2150.

Waters PJ, Parniak MA, Nowacki P, Scriver CR (1998) *In vitro* expression analysis of mutations in phenylalanine hydroxylase: linking genotype to phenotype and structure to function. *Hum Mutat* **11**: 4–17.

Zaremba SM, Gregoret LM (1999) Context-dependence of amino acid residue pairing in antiparallel beta-sheets. *J Mol Biol* **291**: 463–479.

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Convention Planning s.a.s.
Via Fuorimura, 20
80067 Sorrento (Na) – Italy
Phone: +390818071981
Fax: +390818073039
e-mail: homocysteinesorrento@conventionplanning.it

UNITED STATES PATENT AND TRADEMARK OFFICE

EXHIBIT I

CERTIFICATE OF CORRECTION

PATENT NO : 5,763,392
DATED : June 9, 1998
INVENTOR(S) : Barbara C. Hansen, Harry L. Greene, Heidi Ortmeyer

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title Page, Item [73] should read as follows:

Assignee: The University of Maryland, Baltimore. Baltimore, MD

Signed and Sealed this

Twenty-ninth Day of December, 1998

Attest:

Bruce Lehman

Attesting Officer

BRUCE LEHMAN

Commissioner of Patents and Trademarks

US005763392A

United States Patent [19]

Hansen et al.

[11] Patent Number: 5,763,392

[45] Date of Patent: Jun. 9, 1998

[54] TREATMENT OF DIABETES BY ADMINISTRATION OF MYO-INOSITOL

[76] Inventors: Barbara C. Hansen, 6501 Bright Mountain, Mclean, Va. 22101; Harry L. Greene, 777 S. Flager Dr., West Tower Suite 1400, West Palm Beach, Fla. 33401; Heidi K. Ortmeyer, 2 Craven Crt., Baltimore, Md. 21244

[21] Appl. No.: 648,689

[22] Filed: May 15, 1996

Related U.S. Application Data

[63] Continuation of Ser. No. 173,814, Dec. 22, 1993, abandoned.

[51] Int. Cl.[6] A61K 38/00; A01N 43/04; A23L 1/30

[52] U.S. Cl. 514/2; 514/3; 514/52; 514/62; 514/251; 514/725; 514/866; 530/350; 530/399; 530/801; 426/72; 426/74; 426/648; 426/656; 426/658

[58] Field of Search 514/2, 3, 52, 62, 514/251, 725, 866; 530/350, 399; 426/72, 74, 648, 656, 658

[56] References Cited

U.S. PATENT DOCUMENTS

4,921,877	5/1990	Cashmere et al.	514/866
5,124,360	6/1992	Larner et al.	514/738

OTHER PUBLICATIONS

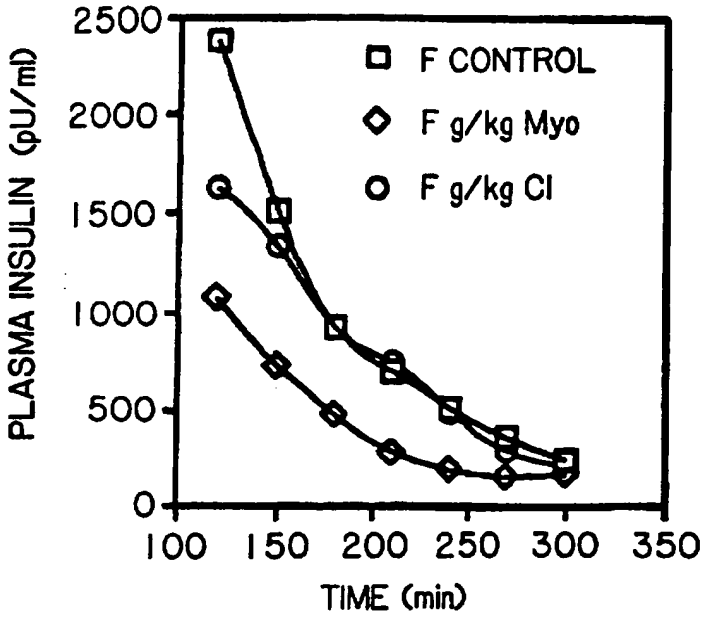
Arendup et al, *Acta Neurol. Scand*, vol. 80, pp. 99–102, 1989.

Primary Examiner—Cecilia J. Tsang
Assistant Examiner—Abdel A. Mohamed

[57] ABSTRACT

Method for lowering the plasma glucose levels of diabetics by administering myo-inositol. Also the invention concerns a nutritional composition containing myo-inositol.

18 Claims, 5 Drawing Sheets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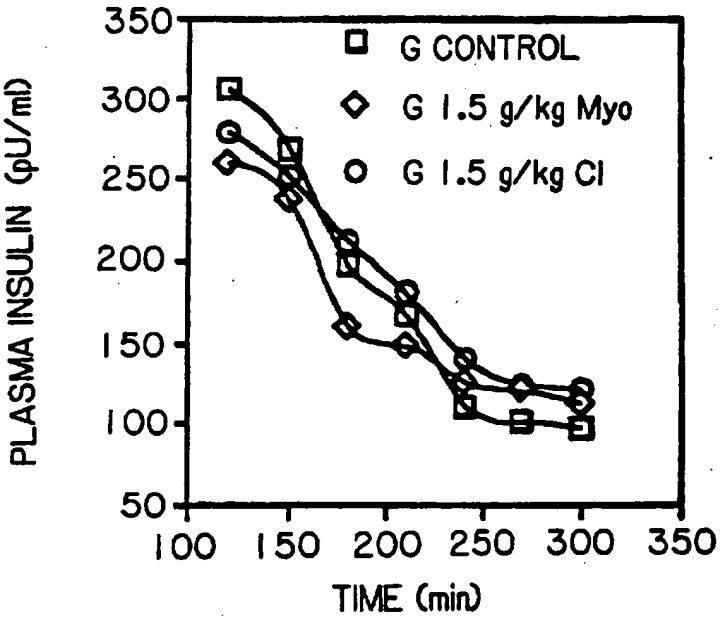
F CONTROL
F g/kg Myo
F g/kg Cl
100 150 200 250 300 350
TIME (min)

FIG. 1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150
100
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G 1.5 g/kg Cl
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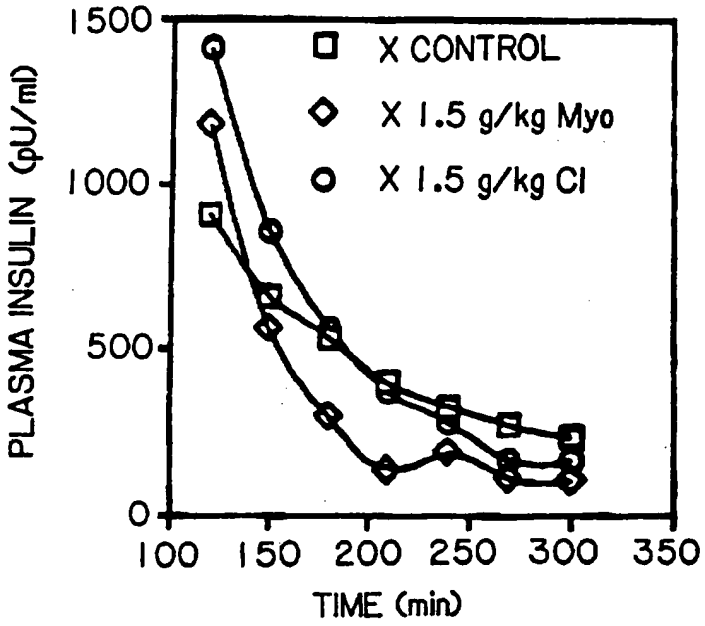
TIME (min)

FIG. 2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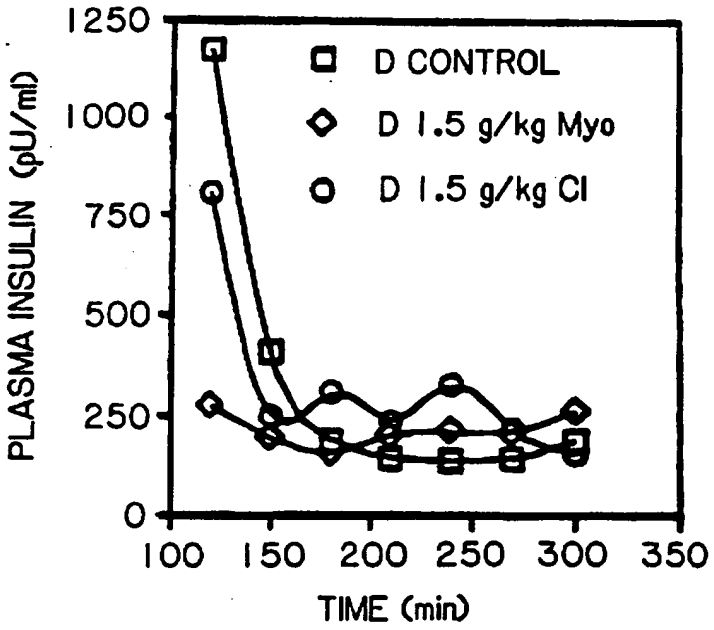
TIME (min)

FIG. 3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TIME (min)

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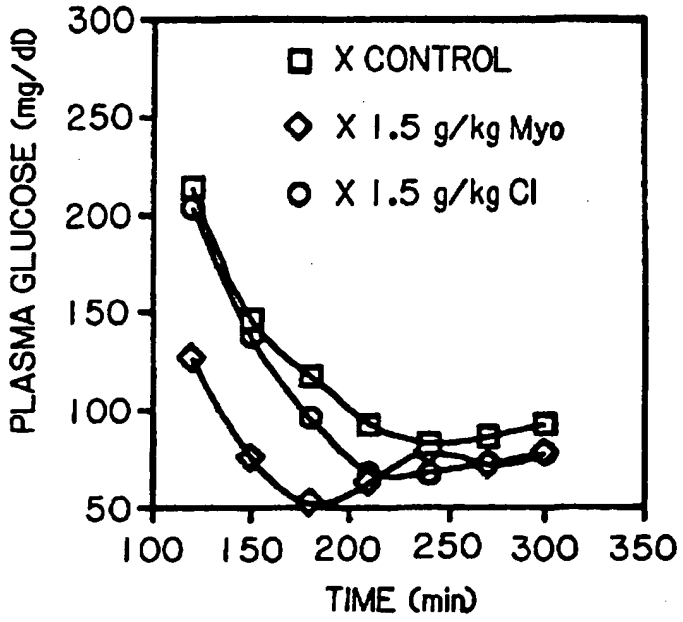
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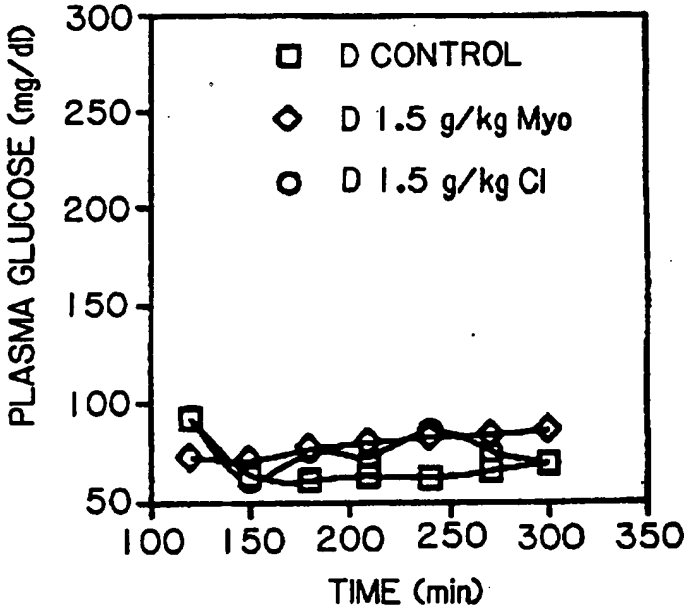
X 1.5 g/kg Myo
X 1.5 g/kg Cl
100 150 200 250 300 350
TIME (min)

FIG. 5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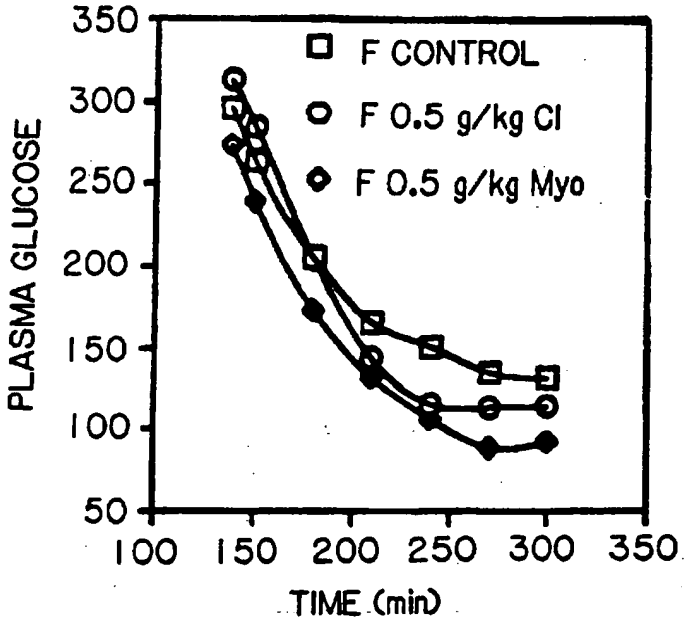
D 1.5 g/kg Myo
D 1.5 g/kg Cl
100 150 200 250 300 350
TIME (min)

FIG. 6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300
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200
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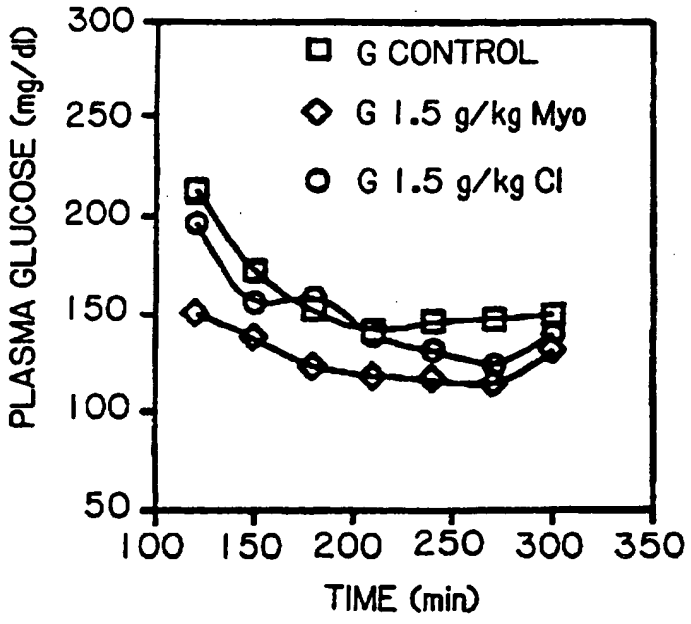
F CONTROL
F 0.5 g/kg Cl
F 0.5 g/kg Myo
100 150 200 250 300 350
TIME (min)

FIG. 7


300
250
200
150
100
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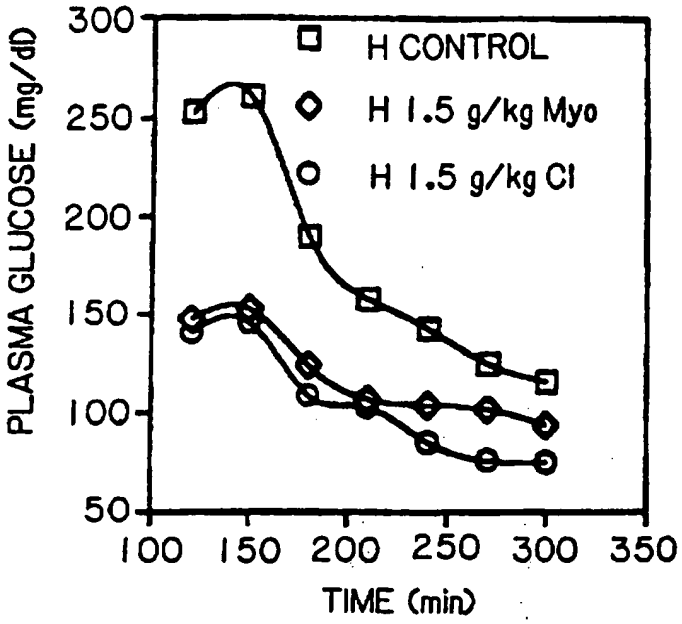
PLASMA GLUCOSE (mg/dl)
G CONTROL
G 1.5 g/kg Myo
G 1.5 g/kg Cl
100 150 200 250 300 350
TIME (min)

FIG. 8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250
200
150
100
50
PLASMA GLUCOSE (mg/dl)
H CONTROL
H 1.5 g/kg Myo
H 1.5 g/kg Cl
100 150 200 250 300 350
TIME (min)

FIG. 9

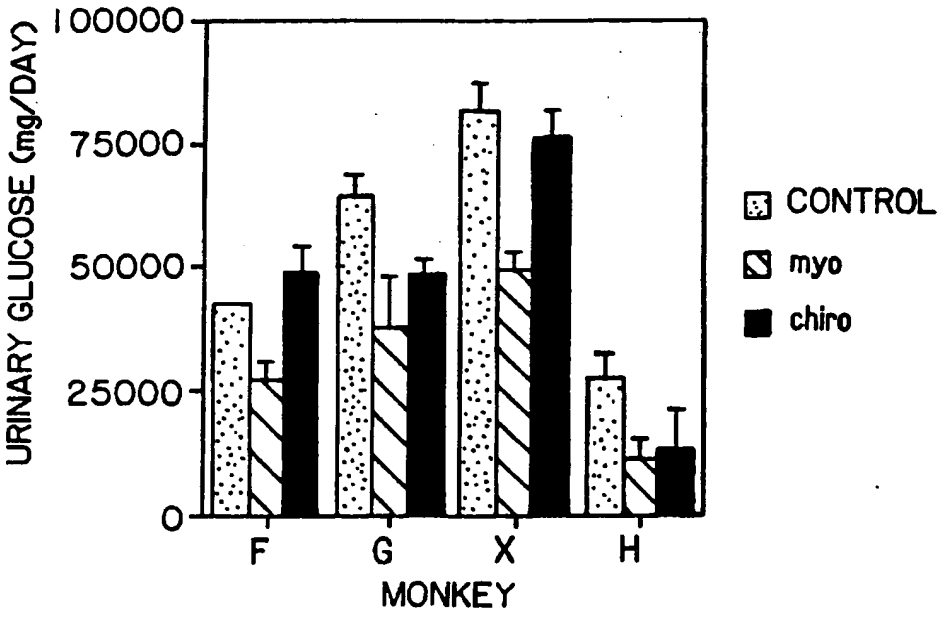
100000
75000
50000
25000
0
URINARY GLUCOSE (mg/DAY)
F
G
X
H
MONKEY
CONTROL
myo
chiro

FIG. 10

TREATMENT OF DIABETES BY ADMINISTRATION OF MYO-INOSITOL

This application is a continuation application of U.S. application Ser. No. 08/173,814, filed Dec. 22, 1993 now abandoned.

FIELD OF THE INVENTION

The present invention concerns treatment of diabetes with myo-inositol and nutritional compositions containing same.

BACKGROUND OF THE INVENTION

Diabetes is a significant disease in humans. Although a complex disease, it is characteristic of subjects having diabetes to have an impaired ability to remove serum or plasma glucose after a glucose load such as after meal ingestion. Such an impairment (sometimes referred to as "glucose intolerance") in severe cases can lead to significant clinical sequelae such as renal failure.

Prior art methods for treating glucose intolerance include the use of insulin or adherence to strict diets which limit intake of easily metabolized carbohydrates. Several liquid enteral formulas designed for use by diabetes patients are commercially available such as Glucerna® nutritional formula (available from Ross Laboratories, Columbus, Ohio). One such enteral formula is described in fructose is part of the carbohydrate component.

Other prior art methods for treating diabetes or glucose intolerance include administration of inositol triphosphate (U.S. Pat. No. 5,023,248) and D-chiro-inositol (U.S. Pat. No. 5,124,360).

Myo-inositol is known in the nutritional field to be an important nutritional component of the diet (see, for example, Holub, B. J., "The Nutritional Significance, Metabolism, and Function of myo-Inositol and Phosphatidylinositol in Health and Disease" in Adv. *Nutr. Res.* 4:107–141 (1982)). Low levels of myo-inositol are typically added to infant formulas and adult enteral nutritional formulas. In addition, administration of low levels of dietary inositol has been found to have certain other beneficial effects (see, Price, D. E., et al., "Effect of Aldose Reductase Inhibition on Nerve Conduction Velocity and Resistance to Ischemic Conduction Block in Experimental Diabetes," *Diabetes*, 37:969–973 (1988); Greene, D. A., et al., "Impaired Rat Sciatic Nerve Sodium-Potassium Adenosine Triphosphatase in Acute Streptozocin Diabetes and its Correction by Dietary Myo-Inositol Supplementation," *J. Clin. Invest.*, 72:1058–1063(1983); Knudsen, G. M., et al., "Myo-Inositol Normalizes Decreased Sodium Permeability of the Blood-Brain Barrier In Streptozotocin Diabetes," *Neuroscience*, 29(3):773–777 (1989); Hallman, M., et al., "Inositol Supplementation In Respiratory Distress syndrome:Relationship Between Serum Concentration, Renal Excretion, and Lung Effluent Phospholipids," *The Journal of Pediatrics*, 110(4):604–610 (1987); and Greene, D. A., et al., "Effects of Insulin and Dietary Myoinositol on Impaired Peripheral Motor Nerve Conduction Velocity in Acute Streptozotocin Diabetes," *The Journal of Clinical Investigation*, 55:1326–1336 (1975)). However, myo-inositol has been reported to be without effect for lowering plasma glucose in streptozotocin diabetic rats after a glucose load (Huang, L. C., et al., "Effect of Acute D-Chiroinositol on Plasma Glucose in Diabetic Rats and Non-Diabetic Rats Given a Glucose Load," *FASEB*, 6(5):A1629, Abstract 4009 (1992)). Furthermore, administration of myo-inositol to patients receiving a high dose (20 g per day) was found not to have any beneficial effects (Arendrup, K., et al., "High-Dose Dietary Myo-Inositol Supplementation Does Not Alter the Ischaemia Phenomenon in Human Diabetes," *Acta Neurol Scand*, 80:99–102 (1978)).

As hereinafter described, it has been unexpectedly discovered that administration of myo-inositol at high doses is effective in lowering plasma glucose levels of diabetics.

SUMMARY OF THE INVENTION

The present invention is directed to a method for treating diabetes in a human subject in need of treatment comprising orally administering to said subject a nutritional composition comprising protein, lipid and carbohydrate in combination with an effective plasma glucose lowering amount of myo-inositol.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 Plasma insulin vs. time in Monkey F for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 2 Plasma insulin vs. time in Monkey G for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 3 Plasma glucose vs. time in Monkey X for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 4 Plasma glucose vs. time in Monkey D for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 5 Plasma glucose vs. time in Monkey X for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 6 Plasma glucose vs. time in Monkey D for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 7 Plasma glucose vs. time in Monkey F for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 8 Plasma glucose vs. time in Monkey G for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 9 Plasma glucose vs. time in Monkey H for the study described in Example 1. "Myo" means myo-inositol and "CI" means D-chiro-inositol.

FIG. 10 Urine glucose (mg/day) for each monkey of the study described in Example 1.

DETAILED DESCRIPTION OF THE INVENTION

In the method of the invention an effective plasma glucose lowering amount of myo-inositol is administered to a subject in need of such treatment. Such subjects are diabetics or patients otherwise manifesting glucose intolerance.

For the purpose of the present invention, the effective amount of myo-inositol to be administered is based on total body mass. Although use of total body mass is more convenient, in some instances it is preferred to use lean body mass because glucose uptake and utilization is primarily a function of lean tissue. Several methods for measuring lean body mass are listed in *Modern Nutrition in Health and Disease*, Ed. Shils, Olson, Shike, 8th Edition, Lea & Febiger, 1994, pages 783–791. Lean body mass can be estimated in humans by the method described as anthropometry (see *Modern Nutrition in Health and Disease*, Ed. Shils, Olson,

Shike. 8th Edition. Lea & Febiger. 1994. pages 785–786). The anthropometry technique uses various body circumferences and skinfold thickness (really a double layer of skin and subcutaneous tissue. measured with special calipers. Satisfactory calipers are Harpenden Caliper. H. E. Morse Co.. Holland. Mich.; Holtain-Harpenden Caliper. Holtain Ltd.. Bymberian. Crymmych. Pembrokeshire. Wales; and Large Caliper. Cambridge Scientific Industries. Inc.. Cambridge. Md.). The usual sites for skinfold measurements are the midtriceps region. at the inferior tip of the scapula. and just above the iliac crest. One grasps the tissue between thumb and forefinger. shaking it gently to exclude underlying muscle. and stretching it just far enough to permit the jaws of the spring-activated caliper to impinge on the tissue. Because the jaws compress the tissue. the caliper reading diminishes for a few seconds. and then the dial is read. In subjects-with moderately firm. rather thin subcutaneous tissue the measurement is easy to make; but in those with flabby. easily compressible tissue and in those with very firm tissue not easily deformable. the measurement is more difficult.

Cross-sectional area of the muscle-bone (M+B) and fat components of the arm can be calculated from arm circumference and skinfold thickness at the midpoint. as follows (T is triceps skinfold. B is biceps):

$$(M+B)\,\text{area} = \frac{1}{4\pi}\left[\text{circ.} - \frac{\pi}{2}(T+BSF)\right]^2,$$

and by subtraction from total area (circ.^{2}/4 π) arm fat area.

An effective amount of myo-inositol is about 0.05 to about 5 g per kg of total body mass per day. preferred is about 0.1 to about 5 g per kg of total body mass per day. more preferred is about 0.2 to about 3 g per kg of total body mass per day. and most preferred is about 0.4 g per kg of total body mass per day.

Myo-inositol for the invention can be obtained from various commercial sources. such as Aldrich Chemical Co.. Milwaukee. Wis. The method of the invention typically results in lowering the plasma glucose level by at least 8%. preferably about 10% to about 50%.

In the method of the invention the myo-inositol is administered in combination with a nutritional composition comprised of protein. lipid. and carbohydrate. Such compositions are typically normal meals. Thus. the myo-inositol can be taken with a meal. just before a meal. or just after a meal. The myo-inositol can be in the form of a tablet. capsule. dry powder. suspension. solution. etc. A preferred means of administering the myo-inositol is to incorporate the myo-inositol into a nutritional composition which is subsequently consumed by the subject.

Thus. the present invention is also directed to a nutritional composition comprising protein. lipid. carbohydrate. and an effective plasma glucose lowering amount of myo-inositol.

The nutritional composition of the present invention is preferably enteral; that is. it is designed for oral. intragastric. or transpyloric use. It is also preferred that the composition is nutritionally complete. By the term "nutritionally complete" is meant that the composition contains adequate nutrients to sustain healthy human life for extended periods. The composition of the invention may be an infant formula or adult nutritional composition. The composition also can be milk-based. soy-based. or based on other nutrients.

The composition of the invention contains ingredients which are designed to meet the nutritional needs of mammals. especially humans. such as a protein (amino acid) source. a lipid source. and a carbohydrate source. The composition of the invention can be an infant or adult nutritional composition. Typically milk. skim milk. casein. hydrolyzed casein. hydrolyzed whey protein. whey. vegetable protein concentrate (e.g. soy protein isolate). hydrolyzed vegetable protein (e.g. soy). animal oils. vegetable oils. starch. sucrose. lactose and/or corn syrup solids will be added to the composition to supply part or all of the amino acids and/or protein. lipid. and carbohydrate as well as other nutrients such as vitamins and minerals.

The composition of the invention preferably comprises about 7% to about 30% protein. about 20% to about 56% lipid. and about 20% to about 70% total carbohydrate. said percentages being based on the total caloric value of the composition. More preferably. the composition of the invention preferably comprises about 10% to about 15% protein. about 35% to about 45% lipid. and about 38% to about 52% total carbohydrate. said percentages being based on the total caloric value of the composition. Most preferably. the composition of the invention preferably comprises about 12% protein. about 40% lipid. and about 45% total carbohydrate. said percentages being based on the total caloric value of the composition.

The amount of myo-inositol in the composition is typically. about 0.25 to about 25 g per 100 calories (cal) of total composition. preferably about 0.5 to about 25 g per 100 cal of total composition. more preferably about 1 to about 15 g per 100 cal of total composition. most preferably about 2 g per 100 cal of total composition. Since the myo-inositol is a significant amount of the total carbohydrates of the diet. it is an advantage of the invention that fewer calories are required for more easily metabolized carbohydrates such as glucose or sucrose.

Other than myo-inositol. the carbohydrate component of the composition of the invention can be any suitable carbohydrate known in the art to be suitable for use in nutritional compositions. Typical carbohydrates include sucrose. fructose. xylitol. glucose. maltodextrin. lactose. corn syrup. corn syrup solids. rice syrup solids. rice starch. modified corn starch. modified tapioca starch. rice flour. soy flour. and the like. It is preferred that part of the carbohydrate is fiber. Examples of suitable fibers include soy fiber. pectin. oat fiber. pea fiber. guar gum. gum acacia. modified cellulose. and the like. The fiber is assumed to not contribute significantly to the caloric content of the composition since fiber is metabolized by the intestinal flora to a variable extent. Fiber preferably comprises about 0.5% to about 4 g per 100 cal of total composition.

The lipid can be any lipid or fat known in the art to be suitable for use in nutritional compositions. Typical lipid sources include milk fat. safflower oil. canola oil. egg yolk lipid. olive oil. cotton seed oil. coconut oil. palm oil. palm kernel oil. soybean oil. sunflower oil. fish oil and fractions of all above oils derived thereof such as palm olein. medium chain triglycerides (MCT). and esters of fatty acids wherein the fatty acids are. for example. arachidonic acid. linoleic acid. palmitic acid. stearic acid. docosahexaeonic acid. eicosapentaenoic acid. linolenic acid. oleic acid. lauric acid. capric acid. caprylic acid. caproic acid. and the like. High oleic forms of various oils are also contemplated to be useful herein such as high oleic sunflower oil and high oleic safflower oil.

The protein can be any protein and/or amino acid mixture known in the art to be suitable for use in nutritional compositions. Typical protein sources are animal protein. vegetable protein such as soy protein. milk protein such as skim milk protein. whey protein and casein. and amino acids (or salts thereof) such as isoleucine. phenylalanine. leucine. lysine. methionine. threonine. tryptophan. arginine.

glutamine, taurine, valine, and the like. A preferred protein source is sodium caseinate or sodium calcium caseinate optionally supplemented with amino acids.

Nutritionally complete compositions contain all vitamins and minerals understood to be essential in the daily diet and these should be present in nutritionally significant amounts. Those skilled in the art appreciate that minimum requirements have been established for certain vitamins and minerals that are known to be necessary for normal physiological function. Practitioners also understand that appropriate additional amounts (overages) of vitamin and mineral ingredients need to be provided to compensate for some loss during processing and storage of such compositions. The composition of the invention preferably contains 100% of the U.S. Recommended Daily Allowance (RDA) in 500 to 3,500 cal of composition, preferably in 500 to 2,000 cal of composition.

To select a specific vitamin or mineral compound to be used in the composition requires consideration of that compound's chemical nature regarding compatibility with the processing and shelf storage.

Examples of minerals, vitamins and other nutrients optionally present in the composition of the invention include vitamin A, vitamin B_6, vitamin B_{12}, vitamin E, vitamin K, vitamin C, folic acid, thiamine, riboflavin, niacin, biotin, pantothenic acid, choline, calcium, phosphorous, iodine, iron, magnesium, copper, zinc, manganese, chloride, potassium, sodium, beta-carotene, nucleotides, selenium, chromium, molybdenum, and L-carnitine. Minerals are usually added in salt form. In addition to compatibility and stability considerations, the presence and amounts of specific minerals and other vitamins will vary somewhat depending on the intended consumer population.

The composition of the invention also typically contains emulsifiers and/or stabilizers such as lecithin, (e.g., egg or soy), carrageenan, xanthan gum, mono- and diglycerides, guar gum, carboxymethyl cellulose, stearoyl lactylates, succinylated monoglycerides, diacetyl tartaric acid esters of monoglycerides, polyglycerol esters of fatty acids, or any mixture thereof.

Artificial sweeteners could also be added to the present composition to enhance the organoleptic quality of the composition. Examples of artificial sweeteners include saccharine, aspartame, and the like.

The composition of the invention may also optionally contain natural or artificial flavorants or colorants such as vanilla, chocolate, coconut, banana, strawberry flavors.

The osmolality of the liquid composition of the invention (when ready to consume) is typically about 100 to about 1100 mOsm/kg H_2O, more typically about 250 to about 700 mOsm/kg H_2O.

The composition of the invention can be prepared by use of standard techniques known in the nutritional art, for example by techniques analogous to those disclosed in U.S. Pat. Nos. 4,921,877 and 4,497,800, the disclosures of which are incorporated herein by reference in their entirety. The myo-inositol can be incorporated into the composition as part of the carbohydrate component or added separately after all other ingredients have been added.

The composition of the invention can be sterilized, if desired, by techniques known in the art, for example, heat treatment such as autoclaving or retorting, irradiation, and the like.

The composition of the invention can be packaged in any type of container known in the art to be useful for storing nutritional products such as glass, lined paperboard, plastic, coated metal cans and the like.

The following examples are to further illustrate the invention but should not be interpreted as a limitation thereof.

EXAMPLE 1

Plasma Glucose Lowering Effect of Myo-Inositol in Rhesus Monkeys

Experimental Design

Chronic administration of myo-inositol and D-chiro-inositol (DCI) incorporated into the complete liquid diet.

1. Compound and administration

Dose: 1.5 g/kg total body mass per day of myo-inositol or DCI for three to five days (in three separate meals).

Route of administration: Oral—incorporated into Sustacal liquid diet (a nutritionally complete liquid diet available from Mead Johnson & Co., Evansville, Ind., U.S.A.) to be ingested by the monkeys as their complete daily provision.

Schedule of administration: Predosing: One to three weeks of Sustacal to establish stable intake levels prior to addition of either DCI or myo-inositol to the diet. Dosing: Three to five days of ingestion of Sustacal liquid diet containing DCI or myo-inositol (blind) followed by one week of Sustacal liquid diet only, then with cross over to three to five days of the alternative agent (given in random order).

Preparation of myo-inositol and DCI compound containing diets: The diets were prepared by careful weighing of doses by weight of the animal and myo-inositol or DCI added to amounts of Sustacal liquid diet calculated to be fully ingested in approximately the first 80% of the food intake for the day (to assure intake of full dose). The research technician was without knowledge of which compound is being added to the diet (wash out period, however, was known due to addition of no agent).

2. Subjects

Rhesus monkeys (Macaca mulatta)—Adult, ages 8 years and over

N=5 monkeys

Monkeys included in this study ranged from hyperinsulinemic with post prandial glycosuria to mildly diabetic. The monkeys are part of a colony maintained by the Obesity, Diabetes and Aging Research Center, University of Maryland. The development of insulin resistance and diabetes in this colony was first described by Hansen, B. C. and Bodkin, N. L. in "Heterogeneity of insulin response: phases leading to type 2 (non-insulin-dependent) diabetes mellitus in the rhesus monkey," *Diabetologia*, 29:713–719 (1986). The monkeys were designated "F", "G", "X", "H" and "D".

3. Outcome measures and effects monitored

a) Food intake measurement:
Sustacal was provided in measured quantity and total food intake per 24 hours determined daily.

b) Body weight:
Was obtained weekly by transfer from cage for weighing (accuracy to 1/10 kg).

c) 24 hour urinary glucose:
Determined quantitatively
1) 3 days during the predosing period
2) 3 days during the period dosing DCI and myo-inositol
Urine was collected directly into an iced container and frozen for glucose analysis.

d) Meal tolerance test
A meal tolerance test was administered on the last day of the DCI dosing period, (with DCI in meal), and on

the last day of the myo-inositol dosing period, (with myo in meal) and compared to baseline meal tolerance tests (Sustacal liquid diet only) obtained prior to initiation of agent. Monkeys were anesthetized, and intravenous cannulas inserted two hours after completion of the meal (15 mls Sustacal/kg body weight). The same volume of Sustacal liquid diet was given for each of the three tolerance tests. Blood samples were obtained at 120, 150, 180, 210, 240, 270 and 300 minutes following the meal. Plasma analyses included glucose and insulin determinations, glucose and insulin disappearance rates and glucose and insulin responses.

4. Statistical tests

Paired t tests were used to identify significant changes between the DCI dosing values and the myo-inositol dosing values with the baseline value.

5. Results

The results of the study are shown in FIGS. 1–10.

FIGS. 1–4 show the data for plasma insulin in four of the monkeys. As can be seen, administration of myo-inositol results in an even lower production of insulin than the control or administration of D-chiro-inositol.

FIGS. 5–9 show the data for plasma glucose in the five monkeys tested. As can be seen, administration of myo-inositol resulted in substantial lowering of the plasma glucose responses to the Sustacal in the four monkeys with an initial elevation in plasma glucose concentration—even more than administration of D-chiro-inositol. All data points (7 samples from each of the 5 monkeys) during the control period were paired with the data points during myo-inositol and DCI consumption and analyzed. Control values were 135±10 mg/dl compared to myo-inositol values of 103±5 mg/dl ($p<0.0001$).

FIG. 10 shows the data for urine glucose for four of the five monkeys. Analysis of paired data from all five monkeys showed that mean urine glucose excretion was significantly reduced from 43±14 g/d during the control period to 25±9 g/d during the period of myo-inositol consumption ($p<0.029$). These data are consistent with the plasma glucose and insulin data.

The meal tolerance test was repeated using 0.5 mg/kg total body weight per day of DCI and myo-inositol. Plasma glucose responses were consistent with the data in FIGS. 5–9.

The results of the study demonstrate that myo-inositol was even more effective than DCI in reducing the plasma glucose levels during the single meal tolerance test as well as the urinary excretion of glucose for the three days during which myo-inositol was a component of the diet.

EXAMPLE 2

A typical nutritionally complete enteral composition of the invention has the following formula:

	100 mL
Calories	106
Protein, g	4.4
Fat, g	4.5
Carbohydrate*, g	12.3
Dietary Fiber, g	1.44
Water, g	85
Vitamin A, IU	420
Vitamin D, IU	34
Vitamin E, IU	6.3
Vitamin K, μg	10.6
Vitamin C, mg	2.5
Folic Acid, μg	34
Thiamine, mg	0.32
Riboflavin, mg	0.36
Niacin, mg	4.2
Vitamin B_6, mg	0.42
Vitamin B_{12} μg	1.27
Biotin, μg	25
Pantothenic Acid, mg	2.1
Choline, mg	42
Calcium, mg	85
Phosphorus, mg	85
Iodine, μg	12.7
Iron, mg	1.52
Magnesium, mg	34
Copper, mg	0.17
Zinc, mg	1.69
Manganese, mg	0.25
Chloride, mg	144 (4.1 mEq)
Potassium, mg	161 (4.1 mEq)
Sodium, mg	93 (4 mEq)
Selenium, μg	8.5
Chromium, μg	8.5
Molybdenum, μg	21
Taurine, mg	12.7
L-Carnitine, mg	19
Myo-inositol, g	12

*excluding myo-inositol

The composition has the following caloric distribution:

Source	% Calories	g/250 Cal (8 fl oz)
Protein sodium and calcium caseinate	17	10.4
Fat partially hydrogenated soy oil MCT oil	37	10.7
Carbohydrate*	46	29

*excluding myo-inositol

I claim:

1. A method for treating diabetes in a human subject comprising orally administering to said subject a human nutritional composition comprising protein, lipid and carbohydrate in combination with an effective plasma glucose lowering amount of myo-inositol of about 0.05 to about 5 g per kg of total body mass per day.

2. The method of claim 1 wherein said effective amount of myo-inositol is about 0.2 to about 3 g per kg of total body mass per day.

3. The method of claim 1 wherein said nutritional composition comprises about 10% to about 15% protein, about 35% to about 45% lipid, and about 38% to about 52% carbohydrate, wherein percentages of said nutritional composition are based on the total caloric value of the composition.

4. The method of claim 1 wherein the plasma glucose level is lowered by at least 8%.

5. The method of claim 1 wherein the plasma glucose level is lowered by about 10% to about 50%.

6. The method of claim 1 further comprising a dietary fiber content of about 0.5 to about 4 g per 100 cal of total composition.

7. The method of claim 1 wherein said nutritional composition comprises about 7% to about 30% protein, about

20% to about 56% lipid, and about 20% to about 70% carbohydrate, wherein percentages of said nutritional composition are based on the total caloric value of the composition.

8. The method of claim 7 wherein the nutritional composition further comprises vitamins and minerals.

9. The method of claim 8 wherein the amount of vitamins and minerals supply 100% of the recommended daily allowance in 500 to 2,500 cal of composition.

10. A nutritional composition comprising protein, lipid, carbohydrate and an effective plasma glucose lowering amount of myo-inositol of about 0.25 to 25 g per 100 cal of total composition.

11. The composition of claim 10 comprising about 7% to about 30% protein, about 20% to about 56% lipid, and about 20% to about 70% total carbohydrate, wherein percentages of said composition are based on the total caloric value of the composition.

12. The composition of claim 10 further comprising dietary fiber content of about 0.5 to about 4 g per 100 cal of total composition.

13. The composition of claim 10 wherein said effective blood glucose lowering amount of myo-inositol is about 1 to about 15 g per 100 cal of total composition.

14. The composition of claim 13 comprising about 10% to about 15% protein, about 35% to about 45% lipid, and about 38% to about 52% total carbohydrate, wherein percentages of said composition are based on the total caloric value of the composition.

15. The composition of claim 13 comprising about 12% protein, about 40% lipid, and about 45% total carbohydrate, said percentages being based on the total caloric value of the composition.

16. The composition of claim 10 further comprising vitamins and minerals.

17. The composition of claim 16 wherein the amount of vitamins and minerals supply 100% of the recommended daily allowance in 500 to 2,000 cal of composition.

18. The composition of claim 17 further comprising at least one of the following components: chromium, selenium, molybdenum, carnitine, taurine, beta-carotene, one or more artificial sweeteners, one or more emulsifiers, or one or more stabilizers.

* * * * *

041-P

PARTIAL AND TOTAL TETRAHYDROBIOPTERIN-RESPONSIVENESS IN CLASSICAL AND MILD PHENYLKETONURIA (PKU)

JB Hennermann[1], B Vetter[1], AE Kulozik[2], E Mönch[1]
[1]Charité Children's Hospital, Humboldt University Berlin, Germany; [2]Children's Hospital, University Hospital Heidelberg, Germany

Hyperphenylalaninemia (HPA) is caused either by a deficiency of the phenylalanine hydroxylase (PAH) or by a defect in the synthesis or regeneration of its coenzyme tetrahydrobiopterin (BH_4). Recently a novel subtype of BH_4-responsive HPA has been reported in several children with milder HPA. These children showed a decrease in serum phenylalanine concentrations after the application of BH_4, but normal BH_4 metabolism and mutations in the PAH gene. We will report 6 further cases of BH_4-responsiveness in children with mild PKU and – for the first time – with classical PKU. BH_4-deficiency was excluded in all children (N. Blau/Zurich). Mutation analysis revealed compound heterozygosity or homozygosity for a mutation in the PAH-gene in all patients. Four of them had not been described before in association with BH_4-sensitive HPA (S110C, D129G, P211T, P281L). The children were treated over a period of 4–13 months (median: 8 months) with BH_4 in a daily dose of 10–20 mg/kg. In children with mild PKU no further dietary treatment was necessary, whereas in children with classical PKU phenylalanine tolerance was significantly elevated. In summary, BH_4 therapy succeeded in an increase of the daily phenylalanine tolerance of 180–1830 mg (median: 1145 mg). These data show, that BH_4-sensitivitiy in PAH-deficiency is not only restricted to mild HPA and may improve phenylalanine tolerance in children with classical PKU as well.

042-P

MILD CLINICAL PRESENTATION OF DIHYDROPTERIDINE REDUCTASE DEFICIENCY IN FOUR SIBLINGS

V Konstantopoulou[1], D Möslinger[2], N Blau[3], W Sperl[4], D Skladal[1]
University Children's Hospitals of [1]Innsbruck and [2]Vienna, Austria, [3]Zürich, Switzerland and [4]Children's Hospital, Salzburg, Austria

Dihydropteridine reductase (DHPR) deficiency is a recessively inherited disorder of tetrahydrobiopterine (BH_4) recycling, that causes a severe deficiency of neurotransmitters in the brain. BH_4 is an essential cofactor for phenylalanine, tyrosine and tryptophan metabolism. Most patients described show severe neurological symptoms (i.e. deterioration of skills, encephalopathy, microcephaly, mental retardation, epilepsy, pyramidal, cerebellar, extrapyramidal signs).

We report four siblings (two girls, two boys; 13.5, 12, 10.5 and 0.7 years) with DHPR deficiency with enzyme activities of 0.3, 0.6, 0.5 and 0.36 mU/mg Hb (normal 2.0–5.0). The index patient (12 years) was found through newborn screening with a phenylalanine concentration of 280 µmol/L (4.7 mg/dl). In the siblings phenylalanine concentrations were below screening detection levels, and were diagnosed because of the index patient, who had decreased concentrations of 5-hydroxy indole acetic acid (median 21 nmol/L, range 5–66; i.e. 4.7 to 63% of lowest normal).

In the first CSF samples, the three siblings showed normal concentrations that later decreased. All four have normal head circumferences (10th–50th percentile). The index patient has a short concentration span and an IQ of 80, but none have severe neurological symptoms, even though because of non-compliance no specific therapy has been given in several years. DHPR deficiency has had a mild clinical course in this family so far. Residual activity of up to 30% seems to be sufficient to prevent severe neurological symptoms, although biochemically very low concentrations of neurotransmitters were observed.

EXHIBIT K

BY JOHN ISAACS, R.PH.

Helping The Medicine Go Down

Tips are offered to assist parents in getting children to swallow their medicine

Darian, age six, cries so hard when he's given his asthma medication that the tablet shoots out of his mouth with his saliva, like a canoe down Niagara Falls—that's the description his mother gives. Emma hides her anticonvulsant capsule under her tongue, then sticks it someplace, like in her poodle's coat. Timmy is 10, but he still gets worked up when he has a tablet or capsule in his mouth, because who can forget what it feels like to have one stuck in the throat? Candice just runs. When caught, she puts her hands over her mouth or buries her mouth in a pillow, cushion or stuffed animal.

Child noncompliance with dosing regimens is a lingering, often serious problem in the delivery of healthcare to children. Difficulty in swallowing medication is common among all age groups, and it is especially prevalent among children and the elderly. Many children have problems swallowing liquids, let alone tablets and capsules. Nearly half of children aged three in the U.S. have been given medication, and it is estimated that half of American children in any two-week period take their medication incorrectly.

Few studies to date have focused on how to equip pharmacists and other healthcare professionals with the skills necessary to teach a child how to swallow medication with relative ease. Yet there are a variety of measures that pharmacists can recommend to parents of children with swallowing difficulties.

One significant dosing problem: When parents struggle to get their children to swallow medication without gagging or spitting it out, it becomes difficult to gauge the amount actually ingested, and some parents actually stop giving the medication in order to not upset the child further.

In many cases, the tablet or capsule is or can be made available in a chewable or liquid form—the pharmacist can even flavor the liquid. Often, the child's caretaker can crush the tablet and dissolve it in a palatable food or beverage.

But liquids require careful administration. Pediatric journals and texts suggest that compliance improves when administering tablets or capsules vs. liquid. Patients comply with taking liquid medications only 60 percent as often as they comply with taking pills.

One compliance issue is in the use of common household teaspoons, which have been shown to vary in capacity from 2.5 to 9 mL, according to a 1975 study by Mattar, Markello and Yaffe.[1] Also, patients and caretakers frequently forget to shake liquid suspensions, jeopardizing proper dosages. In one study, 70 percent of caregivers were unable to demonstrate both an accurately measured and correct liquid dose.[2] The American Association of Poison Control Centers reported in 1996 that more than 30,000 children ages six and under received inappropriate doses of pediatric acetaminophen products. And in the study by Mattar et al, mothers frequently reported that they did not fill the teaspoon, out of concern that the liquid would spill. The taste of liquid medications can be a problem too—some are so unpalatable that they cannot be masked in something edible. In addition, many medications are not commercially available in chewable or liquid form, which brings us back to the pill. The importance—and difficulties—of pill swallowing increases dramatically when treating chronic or serious illnesses. Common strategies of circumvention, such as hiding, crushing or dissolving tablets, may not only affect drug absorption and efficacy, but also circumvent the real problem: The child lacks the necessary skills for swallowing pills.

The pharmacist can direct the parent whose child cannot or will not swallow by suggesting that one of the following be altered: The consistency of material being swallowed; the child's body position; or the method of presentation, which may change the way a child perceives the medication and swallows it.

Water is actually the most difficult liquid for children to swallow.

In addition, the pharmacist may recommend a medical specialist who can introduce the parent to a combination of intervention strategies (relaxation, modeling, shaping, reinforcement) that have been successful in training children to swallow pills. (See sidebar.)

While instructions for taking most medications call for taking them with water, water is actually the most difficult liquid for children to swallow. Thin liquids move quickly in the mouth and do not give the epiglottis time to close fully, thus increasing the chance of choking or aspiration. Thicker liquids or a thicker bolus slow down the oral stage of swallowing—the time the tongue propels the material to the back of the mouth, which initiates the swallowing response. Many infants do fine with formula, for example, but have difficulty swallowing juice. Thus, the pharmacist may suggest to a mother that she add medication to foods with a consistency that the child is used to swallowing safely and easily. For babies, this food is typically rice formula or cereal. For older children, pudding, applesauce, mashed potatoes and yogurt, or making a slit in a Jello cube, may work. Agents such as Thick & Easy, from American Institutional Products, and Thick-It, from Diafoods, make the bolus more thick and dense and may be added to the foods and liquids used to help swallow medication.

Children with *learned* dysphagia can be helped to swallow material of a consistency that they have not managed to swallow before. A child who has had a major choking episode, for example, or a kid who has had trouble swallowing lumps in food, may want to try new foods or a bubble gum-flavored suspension but cannot because he has learned that anything with this consistency has made him "sick" in the past. Certain desensitization techniques, such as that reported by Blount and Dahlquist, may be helpful in these cases. For example, a six-year-old girl was asked to imitate someone who placed and subsequently swallowed a small candy on the back of his tongue. When the child succeeded on the first attempt in two consecutive trials, she was rewarded with praise, hugs and goodies. Then a larger piece of candy was modeled. The process was continued until the child was able to swallow candy the approximate size of the medication prescribed.[3]

Children with low oral tone simply

ART: HARVEY REDDING FOR AMERICAN DRUGGIST

PILL SWALLOWING CAN BE TAUGHT

Two protocols for training pill swallowing have been proposed by Funk, Mullins and Olson, one for children with skills deficit, the other for children who exhibit behavioral noncompliance.[5]

FOR CHILDREN WITH SKILLS DEFICIT, THE FOLLOWING STEPS ARE SUGGESTED:

1. **Relaxation:** In a quiet environment, tell the child to breathe deeply and exhale slowly.
2. **Modeling:** Model swallowing with a candy that can be easily swallowed and demonstrate how to cope with failure (i.e., "I goofed up, but that's okay. I'll give it another try"). Make sure the child understands the difference between practice candy and real medicine.
3. **Practice:** Have the child place candy in his mouth, allow it to dissolve to the size he thinks he can swallow, then swallow. Taking it with water and head tilted can be encouraged. Support the child in swallowing the candy sooner (before it shrinks). Allow the child to progress at his own pace; don't trick or shame the child into swallowing.
4. **Reinforcement:** Reward the child immediately after he swallows the candy.
5. **Repeat:** After two successful trials and reward, introduce slightly larger-size candy and repeat steps two to four. Repeat until approximate pill size is reached.

FOR CHILDREN WHO WON'T SWALLOW PILLS (BEHAVIORAL NONCOMPLIANCE), THESE STEPS ARE PROPOSED:

1. **Reinforcement:** The child is abundantly rewarded and praised in front of family members for promptly taking oral medication at scheduled times. The reason for the reinforcement should be stressed.
2. **Extinction:** The child's attempts to delay administration of medication should be ignored by parents. The child should know why the medication needs to be taken, but an explanation should not need to be repeated with each scheduled dose.
3. **Time Out:** A child who refuses medication should be placed in a nonreinforcing environment for a least 10 minutes. The child is required to be quiet for the last five minutes of this period. Time out should be repeated until child is compliant. Caretakers must remain calm and stick with the program if it is to be successful. All behaviors (hiding, physical resistance) that delay administration of medication must be met with time out or these behaviors will continue and become more frequent.

don't know what to do with a pill in the mouth. Because these children have poor posterior lingual movement, pills stay in their mouths longer, making them gag. Such children, who may have experienced a major choking episode, or who have neurogenic, oral motor or speech problems, can be helped in a number of ways. A therapist may begin with something that the child can swallow, like colored sugar, and then introduce the child to larger foods, like sprinkles, then Tic-Tacs, eventually getting to something that approximates the size of the pill the child needs to swallow. A soft spiked toothbrush, such as Nuk Massage Brush from Gerber, can also be used to wake up oral sensation and increase lingual muscle tone. Pressure applied with the brush against the superior surface of the tongue (blade, dorsum or back) stimulates vertical contraction, and lateral/medial contractions are stimulated by pressure against the tongue's lateral margins (sides).

Kids with low oral tone are often encouraged by therapists to allow the water in their mouths to do the work for them. After placing the pill in his mouth, a child is encouraged to fill his mouth with water until his cheeks are full, and then focus on swallowing the entire bolus in as few swallows as possible.

Or a child may be encouraged to place the pill under his tongue without holding the pill down, relax and drink plenty of water. In both cases, the pill usually slides down easily.

Children with oral hypersensitivity (children who have withstood much suction or dental work) don't like the feel of hard things in their mouths. Children with a history of reflux often don't like texture. Kids who are droolers have a difficult time building the necessary intra-oral pressure to push the pill back and through the esophagus. Thus, when approached by a parent about her child's swallowing inability, it is vital that the pharmacist obtain a detailed child history and refer the parent and child to the dentist, gastroenterologist, speech and language pathologist, or other appropriate medical specialist.

The pharmacist should also inquire about a child's posture when medication is administered. A report in the *British Medical Journal* concluded that because there is a "positive correlation between history of dysphagia, difficulty in swallowing tablets and sensation of tablets sticking (mainly in the throat), and delayed capsule transit," it is important for health professionals to "advise patients to take drugs with a drink while standing" in order to "avoid any local irritant effect of drug contact."[4] For adults and children who do not have swallowing difficulty, standing and tilting the head back is the standard, effective way to swallow a pill (although for light capsules that float on water, a forward head tilt may help to move the capsule to the posterior part of the mouth). However, for smaller children, and children with swallowing difficulty, "the tilted head should be in the same plane as the body," says Maxine Orringer, M.A., CCC-SLP (certificate of clinical competence in speech language pathology) and supervisor, Department of Audiology and Communication Disorders of Pittsburgh's Children's Hospital. "Tipping the child back so the trunk and head are in the same plane will allow him to achieve the benefit of the bolus, of gravity, and help prevent it

from washing into the airway."

For children who drool when drinking, a slight head tilt is best when taking medication. These children have a difficult time keeping their lips together, and thus it's harder for them to build the oral pressure needed to push the pill back and through the esophagus. If a child has unilateral weakness when swallowing, have the child *turn* his head in the direction of the weaker side, so that whatever he is swallowing will go down the stronger side. Or, in order to get the bolus ball into the stronger section of the mouth, have the child with unilateral weakness *tip* his head to the stronger side.

To get the attention of a toddler, advise the parent to put the high chair against a wall. This reduces outside stimulation.

The way medication is presented to children has much to do with their ability and willingness to swallow. Children have preconceived notions based on sight and smell about what they like and do not like. So giving the medication in an opaque cup may do the trick. Or placing a few drops of food coloring (her favorite color) onto the spoon or into the cup may make the medication look more yummy.

Squirting the contents of an oral syringe or dropper into the cheek pouch will help bypass the bitter taste buds on the back of the tongue. The NumiMed medication dispenser, from Sharn of Tampa, FL, helps babies "using natural instincts" to suck only as much from a nipple as they can swallow, reducing the chance of gagging and spitting out medication. The NumiMed bypasses most taste buds and sense of smell so babies take it easily. A strategy for droolers and children with low oral tone, if the child is not a gagger, is to use oral syringes with plungers that bypass the anterior oral cavity and push the medication further back in the mouth.

The Medicine Bottle Co., Hinsdale, IL, markets the Rx Medibottle, which consists of an oral dispenser placed inside a bottle. It offers the accuracy of using an oral dispenser while the bottle makes it easy for a baby to drink.

Some children like to feel they can control how much liquid is tipped and poured into their mouth at one time. Cups with holes on the lid allow a more controlled flow of liquid. For the child who is afraid of choking and would rather tip the cup than tilt his head way back, there are Flexi Cut Cups, Equipment Shop, Bedford, MA, designed to be used with the cut-out side away from the mouth, allowing the child's nose to fit into the cut-out when the cup is tipped for drinking.

There is a rhythm, a stride, to how we eat, chew and swallow. A pharmacist can help parents by making them aware of factors that may cause a deterioration of their child's ability to swallow. Usually, children will chew something to a size that can be swallowed. Children with nasal congestion, however, lose their rhythm when chewing because they are also using their mouths to breath. Saline drops, nasal decongestants or simply blowing the nose before the medication is administered can help the organization of the swallow.

Many children have fatigue dysphagia, even within the course of a meal. A child may swallow well for the first 10 to 15 minutes of a meal, but by the end of the meal he may have a significant disorganization of his swallow. Muscle tone and organization can change with fatigue. For children with progressively labored mealtime swallowing, medication that needs to be taken with food should be given in the middle of the meal and not at the end.

If there is flexibility in terms of when a pill can be administered, then the pharmacist can help parents by asking questions that will help them gauge when during the day pill swallowing will be easiest to achieve. What meal is the child's best, lunch or dinner? Breakfast may be misleading, since swallowing and compliance may seem better because of lighter, more liquid-based foods. When, during the day, does the child pay most attention to you and your directions? Typically, the worst time of day to give medication is at bedtime, when the child is tired and cranky.

An 18-month-old girl who refused to accept pills into her mouth acquired pill-swallowing skills through a procedure called shaping, which is the systematic provision and withholding of reinforcement while the child performs a series of approximations to the desired behavior. For example, the girl was rewarded for touching her cheek with the pill, and later for touching her lips, then teeth and later still for touching her tongue with the pill. She was finally rewarded for placing the pill into her mouth and swallowing it. The procedure required 25 sessions over one week.[3]

Often, a team approach is necessary to assist a child who cannot or will not swallow medication. Specialists may be called upon in speech and language pathology, nutrition, neurology, dentistry, gastrointestinal medicine, radiology, thoracic surgery and otolaryngology-head and neck surgery. The pharmacist, because of his accessibility and knowledge of medications, is likely to be the first healthcare professional approached by a parent whose child is having trouble swallowing liquids and pills. Thus it is vital that the pharmacist obtain a detailed child history, ask the parent significant questions about her child (diet, behavior, health), and, when indicated, recommend those in the team who can help the child swallow the medication that is so vital to his good health. ■

REFERENCES

1. Mattar M.E., Markello J., Yaffe S.J: Inadequacies in the pharmacologic management of ambulatory children. Pediatrics 1975; 87:137-141.
2. Simon H.K, Weinkle D.A.: Over the counter medications, do parents give what they intend to give? Arch Pediatric Adolescent Medicine 1997; 151:654-656.
3. Dahlquist L, Blount R: Teaching a six year old to swallow pills. J Behav Ther Psychiatry 1984; 15:171-173.
4. Channer KS, Virjee J: Effect of posture and drink volume on the swallowing of capsules. British Medical Journal 1982; 285/6356:1702.
5. Wright L, Woodcock J, Scott R: Conditioning children when refusal of oral medication is life threatening. Pediatrics 1969; 44:969-972.
6. Funk MJ, Mullins LL, Olson RA: Teaching children to swallow pills: A case study. Child Health Care 1984; 13:20-23.

Jean Isaacs is a pharmacist, writing instructor and freelance writer in Pittsburgh. He received a B.S. degree in pharmacy and a Master's degree in fine arts in writing from the University of Pittsburgh.

Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency

Shigeo Kure, MD, Dian-Chang Hou, MD, Toshihiro Ohura, MD, Hiroko Iwamoto, MD, Shuhei Suzuki, MD, Naruji Sugiyama, MD, Osamu Sakamoto, MD, Kunihiro Fujii, MD, Yoichi Matsubara, MD, and Kuniaki Narisawa, MD

Serum phenylalanine concentrations decreased in 4 patients with hyperphenylalaninemia after loading with tetrahydrobiopterin. There were no abnormalities in urinary pteridine excretion or in dihydropteridine reductase activity. However, mutations were detected in the phenylalanine hydroxylase gene, suggesting a novel subtype of phenylalanine hydroxylase deficiency that may respond to treatment with cofactor supplementation.
(J Pediatr 1999;135:375-8)

Hyperphenylalaninemia is caused by a deficiency of either phenylalanine hydroxylase or its cofactor, tetrahydrobiopterin.[1] Accurate differentiation is required because patients with the latter disorder must be treated with the cofactor and/or neurotransmitter as early as possible to minimize irreversible brain damage. BH4 and combined phenylalanine and BH4 loading tests are currently used for differentiation, because the serum Phe concentration decreases after BH4 administration in BH4 deficiency, but not in PAH deficiency.[2-4]

Recently, we encountered patients with mild HPA whose elevated serum Phe concentrations gradually decreased after oral administration of BH4. Urinary pteridines and dihydropteridine reductase activities were normal, suggesting that the patients were deficient in PAH rather than BH4. Because our results differed from published reports showing that patients with PAH deficiency do not respond to BH4, the nature of the metabolic defect was not clear. Molecular analysis of the *PAH* gene showed that mutations were present in all the alleles.

From the Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Miyagi, Japan; Department of Pediatrics, Tohoku University School of Medicine, Sendai, Miyagi, Japan; Division of Pediatric Neurology, Kanagawa Children's Medical Center, Yokohama, Kanagawa, Japan; Department of Pediatrics, Osaka Medical College, Takatsuki, Osaka, Japan; and Department of Pediatrics, Yokkaichi Municipal Hospital, Yokkaichi, Mie, Japan.

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Reprint requests: Shigeo Kure, MD, Department of Medical Genetics, Tohoku University School of Medicine, 1-1 Seiryomachi, Aobaku, Sendai, 980-8574, Japan.

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METHODS

Patients

At neonatal screening the serum Phe concentrations of patients 1 to 5 were 16, 4, 10, 12, and 16 mg/dL, respectively. All patients were treated with a low-Phe diet (Phe, 50-60 mg/kg/d) to maintain a serum Phe level below 4 mg/dL. All were considered to have a mild form of HPA because their serum Phe concentrations never exceeded 20 mg/dL, even when they were not on the Phe-restricted diet.

BH4 Tetrahydrobiopterin
DHPR Dihydropteridine reductase
HPA Hyperphenylalaninemia
PAH Phenylalanine hydroxylase
Phe Phenylalanine
PKU Phenylketonuria

Biochemical Analyses

Urinary pteridine compounds were analyzed by high-performance liquid chromatography.[5] DHPR activity was measured in Guthrie card specimens as described.[6]

BH4 Loading Test

Patients received non-Phe-restricted meals 2 days before initiating the BH4 loading test and during the test. The estimated Phe intake during the test was 80 to 90 mg/kg per day. BH4 (Suntory, Tokyo, Japan) was administered orally in a loading test at a dose of 5 or 10 mg/kg, and blood samples were obtained at least 2 hours after each meal. Serum Phe concentrations were determined by using an automated amino acid analyzer (L-8500; Hitachi, Hitachi, Japan).

Mutational Analysis of PAH Gene

Seven mutations prevalent in Asian patients with classical phenylketonuria

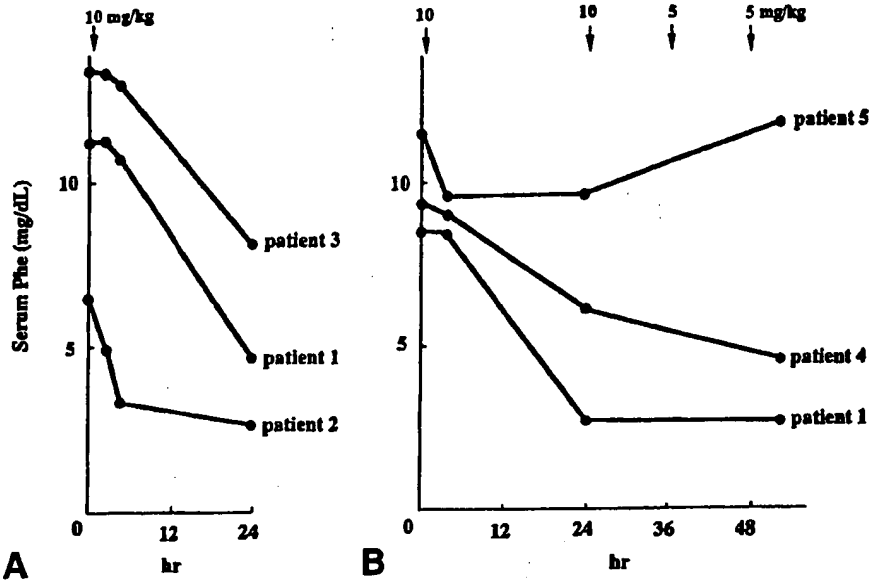

Figure. BH4 loading test in patients with PAH deficiency. **A,** Conventional protocol. *Arrow* indicates time of oral BH4 administration (10 mg/kg body weight). Blood samples were collected to determine serum Phe concentrations at 0, 2, 4, and 24 hours after initiation of loading. Results are for patients 1, 2, and 3 during infancy. **B,** Modified protocol. BH4 was administered orally 4 times. *Arrows* indicate times of BH4 administration at 0 (10 mg/kg), 24 (10 mg/kg), 36 (5 mg/kg), and 48 hours (5 mg/kg). Blood samples were obtained at 0, 4, 24, and 52 hours. These tests were performed in patients 1, 4, and 5 at 1.3, 13, and 8 years of age, respectively.

were screened by allele-specific amplification. These mutations included R111X, IVS4-1G→A, Y204C, R241C, R243Q, Y356X, and R413P. Other mutations were identified by analyzing a minute amount of ectopically expressed PAH messenger RNA in lymphoblasts established from our patients. Briefly, the entire coding region of PAH complementary DNA was amplified by reverse-transcription–mediated nested polymerase chain reaction. Nucleotide sequences of the amplified cDNA fragments were directly determined by using a ThermoSequenase cycle sequencing kit (Amersham, Little Chalfont, England) and the A.L.F. automated DNA sequencer (Pharmacia, Uppsala, Sweden).

RESULTS

Serum Phe concentrations were analyzed after oral administration of BH4, 10 mg/kg body weight, to 3 patients with HPA (Figure, *A*). Serum Phe concentrations gradually decreased, never falling to the normal range for Phe (1 to 2 mg/dL). This type of response contrasted sharply with findings from patients with a deficiency in the BH4 synthesizing system (such as that of 6-pyruvoyltetrahydropterin synthetase), in which the serum Phe concentration is typically normalized within 2 to 4 hours of administration.[8]

The decrease of serum Phe concentrations in our patients apparently did not reach the lowest level at 24 hours (Figure, *A*). To evaluate the extent to which the serum Phe concentration decreased, we modified the BH4 loading protocol. The monitoring period was extended from 24 to 52 hours. Because the half-life of orally administered BH4 in serum was 1.1 and 3.5 hours in rats[9] and humans (Suntory Co Ltd, unpublished data), respectively, BH4 was administered again at 24 hours (10 mg/kg body weight) and at 36 and 48 hours (5 mg/kg body weight) to maintain high plasma BH4 levels during the loading test. Patient 1 was re-evaluated according to this modified protocol at the age of 1.3 years. We were unable to obtain parental consent to perform the modified loading test on patients 2 and 3. The serum Phe concentration gradually decreased and remained low for 24 hours (Figure, *B*). These findings indicate that the response of patient 1 to BH4 was reproducible and excluded the possibility of a transient neonatal form of BH4 deficiency.[10]

A gradual response has also been found in some patients with a DHPR deficiency when a low dose of BH4 was used in the loading test.[8] Urinary pteridine analysis disclosed neopterin/total pterin ratios of 48%, 53%, and 50% in patients 1, 2, and 3, respectively (normal, 27% to 62%). DHPR activities in Guthrie card specimens from patients 1, 2, and 3 were 0.89, 0.81, and 0.94 nmol of cytochrome *c* reduced per minute per 3-mm diameter filter disk, respectively (normal, 0.8 to 1.2). These 3 patients were therefore unlikely to have biochemical abnormalities in the BH4 metabolic pathway.

Patients with a PAH deficiency that responds to BH4 have not been described. We therefore analyzed our patients further by mutational analysis of the *PAH* gene (Table). Five mutations (R252W[1], IVS4-1G→A[1], R413P[1], R241C[11], and P407S[12]) have been found in patients with classical PKU. The A373T mutation has not been described. Identification of a mutation in each *PAH* gene allele indicated that patients 1 to 3 had a PAH deficiency. Homozygotes of R252W, IVS4-1G→A, and R413P presented with clinical symptoms typical of classical PKU. Therefore these mutations appear to abolish PAH function. In contrast, the serum Phe concentrations of our patients never exceeded 20 mg/dL, even when they were not on a Phe-restricted diet, suggesting that mutant PAH molecules with P407S, A373T, and R241C have residual enzymatic activities.

If responsiveness to BH4 is determined by the nature of mutations, patients who share identical mutations should respond similarly to BH4. To test this notion, we performed a BH4

Table. Mutations in the *PAH* gene identified in patients with HPA

Patient No.	Allele	Mutation code	Nucleotide change*	Effect on coding
1	1	P407S	C1441T in exon 12	Pro→Ser at codon 407
	2†	R252W	C976T in exon 7	Arg→Trp at codon 252
2	1	IVS4-1G→A	gt→at splicing donor site in intron 4	Splicing defect
	2	A373T	G1339A in exon 11	Ala→Thr at codon 373
3	1	R413P	G1460C in exon 12	Arg→Pro at codon 413
	2	R241C	C943T in exon 7	Arg→Cys at codon 241
4	1	R413P	G1460C in exon 12	Arg→Pro at codon 413
	2	R241C	C943T in exon 7	Arg→Cys at codon 241
5	1	P407S	C1441T in exon 12	Pro→Ser at codon 407
	2	R111X	C563T in exon 3	Premature termination at codon 111

Pro, Proline; *Ser*, serine; *Arg*, arginine; *Trp*, tryptophan; *Ala*, alanine; *Thr*, threonine; *Cys*, cysteine.
*Numbered according to Kwok et al (1985).[15]
†Novel mutation identified in this study.

loading test in patient 4, whose mutations (R241C and R413P) were identical to those in patient 3 (Table). Patient 4 was an 8-year-old boy with no known relationship to patient 3. The serum Phe concentration in patient 4 decreased in response to BH4 in a manner similar to that in patient 3 (Figure, *B*). We then examined the response to BH4 of patient 5, a 13-year-old girl with HPA. She shared the P407S mutation with patient 1 but had a nonsense mutation, R111X, instead of the R413P mutation (Table). Her HPA was refractory to BH4 supplementation; the serum Phe concentration decreased only slightly, returning within 36 hours to the level before loading (Figure, *B*).

DISCUSSION

The mechanism of BH4 responsiveness may be explained by distinct mutations in the *PAH* gene. Normal human PAH is present as a homotetramer or a homodimer.[13] In patient 1, who responded well to BH4 therapy, P407S and R252W subunits should associate to form various P407S/R252W heteropolymers in addition to P407S and R252W homopolymers. Patient 5 shared only one mutant allele (*P407S*) with patient 1 and responded poorly to BH4. The other *R111X* allele is supposed to generate a truncated PAH subunit that is unlikely to associate with the P407S subunit. These observations suggested that the composition of the PAH subunits may be important for BH4 responsiveness. Namely, P407S/R252W heteropolymers or R252W homopolymers, but not P407S homopolymers, probably form mutant PAH with a high Michaelis-Menten constant K_m for BH4. It is likely that BH4 supplementation increased the intracellular BH4 concentration to restore residual PAH activity and/or to stabilize the mutant PAH molecules. Likewise, R413P/R241C heteropolymers may constitute BH4-responsive PAH subunits in patients 3 and 4.

Our results identified a novel subtype of PAH deficiency and suggest the therapeutic potential of BH4. Clinical outcomes in HPA are sometimes unsatisfactory because of limited compliance with a strict Phe-restricted diet. To date, no supportive therapy has been established to allow a diet less restrictive in Phe. Micro-encapsulated Phe lyase[14] and gene therapy are currently under development, but neither is presently available for clinical use. Although BH4 therapy is not always effective in PAH deficiency, it may be beneficial to a subgroup of patients with specific PAH mutations. Indeed, the oral administration of BH4, maintained sufficiently, allowed low serum Phe concentrations in patient 1 for over 24 hours without a Phe-restricted diet (Figure, *B*). All patients studied here had mild HPA, representing non-PKU HPA. However, some individuals with PKU might be partially responsive to BH4. Further studies of patients with HPA associated with various mutations are necessary to evaluate the therapeutic potential of BH4 in PAH deficiency.

REFERENCES

1. Scriver CR, Kaufman S, Eisensmith RC, Woo SLC. The hyperphenylalaninemias. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular basis of inherited disease. New York: McGraw-Hill, Inc; 1995. p. 1015-76.
2. Danks DM, Cotton RGH, Schlesinger P. Diagnosis of malignant hyperphenylalaninemia. Arch Dis Child 1979;54: 329-30.
3. Guttler F, Lou H, Lykkelund C, Niederwieser A. Combined tetrahydrobiopterin-phenylalanine loading test in the detection of partially defective biopterin synthesis. Eur J Pediatr 1984;142:126-9.
4. Ponzone A, Guardamagna O, Spaga M, Ferraris S, Ponzone R, Kierat L, et al. Differential diagnosis of hyperphenylalaninemia by a combined phenylalanine-tetrahydrobiopterin loading test. Eur J Pediatr 1993;152:655-61.

5. Fukushima T, Nixon JC. Analysis of reduced forms of biopterin in biological tissues and fluids. Anal Biochem 1980;102:176-88.
6. Arai N, Narisawa K, Hayakawa H, Tada K. Hyperphenylalaninemia due to dihydropteridine reductase deficiency: diagnosis by enzyme assay on dried blood spots. Pediatrics 1982;98:426-30.
7. Takahashi K, Kure S, Matsubara Y, Narisawa K. Novel phenylketonuria mutation detected by analysis of ectopically transcribed phenylalanine hydroxylase mRNA from lymphoblast [abstract]. Lancet 1992;340:1473.
8. Ponzone A, Guardamagna O, Ferraris S, Ferrero GB, Dianzani I, Cotton RG. Tetrahydrobiopterin loading test in hyperphenylalaninemia. Pediatr Res 1991;30:435-8.
9. Hayashi T, Ogata A, Takehisa M, Komoridani K, Oonuma N. Studies on metabolism and disposition of sapropterine hydrochloride (SUN-0588) L-erythro-tetrahydrobiopterin hydrochloride in rats. Clin Report 1992;26:3471-95.
10. Takahashi T, Kodama S, Nishio H, Takumi T, Matsuo T, Hase Y, et al. Transient hyperphenylalaninemia with a high neopterin to biopterin ratio in urine. J Inherit Metab Dis 1985;8:105-8.
11. Takarada Y, Kalanin J, Yamashita K, Ohtsuka N, Kagawa S, Matsuoka A. Phenylketonuria mutant alleles in different populations: missense mutation in exon 7 of phenylalanine hydroxylase gene. Clin Chem 1993;39:2354-5.
12. Perez B, Desviat LR, Ugarte M. Analysis of the phenylalanine hydroxylase gene in the Spanish population: mutation profile and association with intragenic polymorphic markers. Am J Hum Genet 1997;60:95-102.
13. Kaufman S, Fisher DB. Purification and some physical properties of phenylalanine hydroxylase from rat liver. J Biol Chem 1970;245:4745-50.
14. Chang TM, Bourget L, Lister C. A new theory of enterorecirculation of amino acids and its use for depleting unwanted amino acids using oral enzyme-artificial cells, as in removing phenylalanine in phenylketonuria. Artif Cells Blood Substit Immobil Biotechnol.1995;23:1-21.
15. Kwok SC, Ledley FD, DiLella AG, Robson KJ, Woo SL. Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase. Biochemistry 1985;24:556-61.

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(12) **United States Patent**
Mukerji et al.

(10) **Patent No.: US 6,428,990 B1**
(45) **Date of Patent: Aug. 6, 2002**

EXHIBIT M

(54) **HUMAN DESATURASE GENE AND USES THEREOF**

(75) Inventors: **Pradip Mukerji**; **Amanda Eun-Yeong Leonard**, both of Gahanna; **Yung-Sheng Huang**, Columbus; **Jennifer M. Parker-Barnes**, New Albany, all of OH (US)

(73) Assignee: **Abbott Laboratories**, Abbott Park, IL (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/439,261**

(22) Filed: **Nov. 12, 1999**

Related U.S. Application Data

(63) Continuation-in-part of application No. 09/227,613, filed on Jan. 8, 1999, which is a continuation-in-part of application No. PCT/US98/07422, filed on Apr. 10, 1998, which is a continuation-in-part of application No. 08/833,610, filed on Apr. 11, 1997, now Pat. No. 5,972,664.

(51) **Int. Cl.**[7] **C12P 7/64**; C12P 7/62; C12N 9/02; C12N 1/20; C07H 21/04

(52) **U.S. Cl.** **435/134**; 435/135; 435/136; 435/189; 435/252.3; 435/320.1; 536/23.2; 530/350

(58) **Field of Search** 435/189, 252.3, 435/320.1, 134–136; 536/23.2; 530/350

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,443,974	A	8/1995	Hitz et al.	435/172.3
5,552,306	A	9/1996	Thomas et al.	435/134

FOREIGN PATENT DOCUMENTS

WO	WO 93/11245	6/1993
WO	WO 94/11516	5/1994
WO	WO 96/13591	5/1996
WO	9846765	10/1998
WO	0020603	4/2000

OTHER PUBLICATIONS

Sequence Search Alignment of Applicants' SEQ ID No. : 1 and prior art sequence in Chaudhary et al. [WO9846763–A1, Oct. 22, 1998].*

Lamerdin J. E. et al.: "BC269730_2" EMBL Database Entry 060427; Accession No. 060427 Aug. 1, 1998, XP002140846.

Cho H. P. et al.: "Cloning, Expression, and Fatty Acid Regulation of the Human Delta–5 Desaturase" Journal of Biological Chemistry, vol. 274, No. 52, Dec. 24, 1999, pp. 37335–37339, XP002140847.

Michaelson L. et al.: "Isolation of a delta5–fatty acid desaturase gene from *Mortierella alpina*" Journal of Biological Chemistry, vol. 273, No. 30, Jul. 24, 1998, pp. 19055–19059, XP002076636.

The Faseb Journal, Abstracts, Part I, Abstract 3093, p. A532 (Experimental Biology 98, San Francisco, CA, Apr. 18–22, 1998).

Deborah S. Knutzon et al.—"Identification of 5–Desaturase from *Mortierella alpina* by Heterologous Expression in Bakers' Yeast and Canola" The Journal of Biological Chemistry (1998) vol. 273, No. 45, Issue of Nov. 6, pp. 29360–29366 (1998).

Hyekyung P. Cho, et al.,—"Cloning, Expression, and Nutritional Regulation of the Mammalian –6 Desaturase" The Journal of Biological Chemistry (1999) vol. 274, No.1, Issue of Jan. 1, pp. 471–477 (1999).

J. E. Lamerdin, et al., "Sequence Analysis of a Human BAC Containing the FEN1 DNA Repair Gene" —Accession AC004770, Jun. 12, 1998—GenBank.

* cited by examiner

Primary Examiner—Tekchand Saidha
(74) *Attorney, Agent, or Firm*—Cheryl L. Becker

(57) **ABSTRACT**

The subject invention relates to the identification of a gene involved in the desaturation of polyunsaturated fatty acids at carbon 5 (i.e., "human Δ5-desaturase") and to uses thereof. In particular, human Δ5-desaturase may be utilized, for example, in the conversion of dihomo-γ-linolenic acid (DGLA) to arachidonic acid (AA) and in the conversion of 20:4n-3 to eicosapentaenoic acid (EPA). AA or polyunsaturated fatty acids produced therefrom may be added to pharmaceutical compositions, nutritional compositions, animal feeds, as well as other products such as cosmetics.

6 Claims, 48 Drawing Sheets

Sections of the Desaturases	Clone ID from Incyte LifeSeq Database	Keyword
151-300 delta 5	3808675	fatty acid desaturase
301-446 delta 5	354535	delta 6
151-300 delta 6	3448789	delta 6
151-300 delta 6	1362863	delta 6
151-300 delta 6	2394760	delta 6
301-457 delta 6	3350263	delta 6

FIG. 1

Edited Contig 2692004

GCACGCCGACCGGCGCCGGGAGATCCTGGCAAAGTATCCAGAGATAAAGTCCTTGATGAAACCTGATCCCAAT
TTGATATGGATTATAATTATGATGGTTCTCACCCAGTTGGGTGCATTTTACATAGTAAAAGACTTGGACTGGA
AATGGGTCATATTTGGGGCCTATGCGTTTGGCAGTTGCATTAACCACTCAATGACTCTGGCTATTCATGAGAT
TGCCCACAATGCTGCCTTTGGCAACTGCAAAGCAATGTGGAATCGCTGGTTTGGAATGTTTGCTAATCTTCCT
ATTGGGATTCCATATTCAATTTCCTTTAAGAGGTATCACATGGATCATCATCGGTACCTTGGAGCTGATGGCG
TCGATGTAGATATTCCTACCGATTTTGAGGGCTGGTTCTTCTGTACCGCTTTCAGAAAGTTTATATGGGTTAT
TCTTCAGCCTCTCTTTTATGCCTTTCGACCTCTGTTCATCAACCCCAAACCAATTACGTATCTGGAAGTTATC
AATACCGTGGCACAGGTCACTTTTGACATTTTAATTTATTACTTTTTGGGAATTAAATCCTTAGTCTACATGT
TGGCAGCATCTTTACTTGGCCTGGGTTTGCACCCAATTTCTGGACATTTTATAGCTGAGCATTACATGTTCTT
AAAGGGTCATGAAACTTACTCATATTATGGGCCTCTGAATTTACTTACCTTCAATGTGGGTTATCATAATGAA
CATCATGATTTCCCCAACATTCCTGGAAAAAGTCTTCCACTGGTGAGGAAAATAGCAGCTGAATACTATGACA
ACCTCCCTCACTACAATTCCTGGATAAAAGTACTGTATGATTTTGTGATGGATGATACAATAAGTCCCTACTC
AAGAATGAAGAGGCACCAAAAAGGAGAGATGGTGCTGGAGTAAATATCATTAGTGCCAAAGGGATTCTTCTCC
AAAACTTTAGATGATAAAATGGAATTTTTGCATTATTAAACTTGAGACCAGTGATGCTCAGAAGCTCCCCTGG
CACAATTTCAGAGTAAGAGCTCGGTGATACCAAGAAGTGAATCTGGCTTTTAAACAGTCAGCCTGACTCTGTA
CTGCTCAGTTTCACTCACAGGAAACTTGTGACTTGTGTATTATCGTCATTGAGGATGTTTCACTCATGTCTGT
CATTTTATAAGCATATCATTTAAAAAGCTTCTAAAAAGCTATTTCGCCAGG

FIG.2

Edited Contig 2153526

TTACCTTCTACGTCCGCTTCTTCCTCACTTATGTGCCACTATTGGGGCTGAAAGCTTCCTGGGCCTTTTCTTC
ATAGTCAGGTTCCTGGAAAGCAACTGGTTTGTGTGGGTGACACAGATGAACCATATTCCCATGCACATTGATC
ATGACCGGAACATGGACTGGGTTTCCACCCAGCTCCAGGCCACATGCAATGTCCACAAGTCTGCCTTCAATGA
CTGGTTCAGTGGACACCTCAACTTCCAGATTGAGCACCATCTTTTTCCCACGATGCCTCGACACAATTACCAC
AAAGTGGCTCCCCTGGTGCAGTCCTTGTGTGCCAAGCATGGCATAGAGTACCAGTCCAAGCCCCTGCTGTCAG
CCTTCGCCGACATCATCCACTCACTAAAGGAGTCAGGGCAGCTCTGGCTAGATGCCTATCTTCACCAATAACA
ACAGCCACCCTGCCCAGTCTGGAAGAAGAGGAGGAAGACTCTGGAGCCAAGGCAGAGGGGAGCTTGAGGGACA
ATGCCACTATAGTTTAATACTCAGAGGGGGTTGGGTTTGGGGACATAAAGCCTCTGACTCAAACTCCTCCCTT
TTATCTTCTAGCCACAGTTCTAAGACCCAAAGTGGGGGGTGGACACAGAAGTCCCTAGGAGGGAAGGAGCT

FIG.3

Edited Contig 3506132

GTCTTTTACTTTGGCAATGGCTGGATTCCTACCCTCATCACGGCCTTTGTCCTTGCTACCTCTCAGGCCCAAG
CTGGATGGCTGCAACATGATTATGGCCACCTGTCTGTCTACAGAAAACCCAAGTGGAACCACCTTGTCCACAA
ATTCGTCATTGGCCACTTAAAGGGTGCCTCTGCCAACTGGTGGAATCATCGCCACTTCCAGCACCACGCCAAG
CCTAACATCTTCCACAAGGATCCCGATGTGAACATGCTGCACGTGTTTGTTCTGGGCGAATGGCAGCCCATCG
AGTACGGCAAGA

FIG.4

Edited Contig 3854933

CAGGGACCTACCCCGCGCTACTTCACCTGGGACGAGGTGGCCCAGCGCTCAGGGTGCGAGGAGCGGTGGCTAG
TGATCGACCGTAAGGTGTACAACATCAGCGAGTTCACCCGCCGGCATCCAGGGGGCTCCCGGGTCATCAGCCA
CTACGCCGGGCAGGATGCCACGGATCCCTTTGTGGCCTTCCACATCAACAAGGGCCTTGTGAAGAAGTATATG
AACTCTCTCCTGATTGGAGAACTGTCTCCAGAGCAGCCCAGCTTTGAGCCCACCAAGAATAAAGAGCTGACAG
ATGAGTTCCGGGAGCTGCGGGCCACAGTGGAGCGGATGGGGCTCATGAAGGCCAACCATGTCTTCTTCCTGCT
GTACCTGCTGCACATCTTGCTGCTGGATGGTGCAGCCTGGCTCACCCTTTGGGTCTTTGGGACGTCCTTTTTG
CCCTTCCTCCTCTGTGCGGTGCTGCTCAGTGCAGTTCAGGCCCAGGCTGGCTGGCTGCAGCATGACTTTGGGC
ACCTGTCGGTCTTCAGCACCTCAAAGTGGAACCATCTGCTACATCATTTTGTGATTGGCCACCTGAAGGGGGC
CCCCGCCAGTTGGTGGAACCACATGCACTTCCAGCACCATGCCAAGCCCAACTGCTTCCGCAAAGACCCAGAC
ATCAACATGCATCCCTTCTTCTTTGCCTTGGGGAAGATCCTCTCTGTGGAGCTTGGGAAACAGAAGAAAAAAT
ATATGCCGTACAACCACCAGCACARATACTTCTTCCTAATTGGGCCCCAGCCTTGCTGCCTCTCTACTTCCA
GTGGTATATTTTCTATTTTGTTATCCAGCGAAAGAAGTGGGTGGACTTGGCCTGGATCAGCAAACAGGAATAC
GATGAAGCCGGGCTTCCATTGTCCACCGCAAATGCTTCTAAA

FIG.5

Edited Contig 2511785

GCCACTTAAAGGGTGCCTCTGCCAACTGGTGGAATCATCGCCACTTCCAGCACCACGCCAAGCCTAACATCTT
CCACAAGGATCCCGATGTGAACATGCTGCACGTGTTTGTTCTGGGCGAATGGCAGCCCATCGAGTACGGCAAG
AAGAAGCTGAAATACCTGCCCTACAATCACCAGCACGAATACTTCTTCCTGATTGGGCCGCCGCTGCTCATCC
CCATGTATTTCCAGTACCAGATCATCATGACCATGATCGTCCATAAGAACTGGGTGGACCTGGCCTGGGCCGT
CAGCTACTACATCCGGTTCTTCATCACCTACATCCCTTTCTACGGCATCCTGGGAGCCCTCCTTTTCCTCAAC
TTCATCAGGTTCCTGGAGAGCCACTGGTTTGTGTGGGTCACACAGATGAATCACATCGTCATGGAGATTGACC
AGGAGGCCTACCGTGACTGGTTCAGTAGCCAGCTGACAGCCACCTGCAACGTGGAGCAGTCCTTCTTCAACGA
CTGGTTCAGTGGACACCTTAACTTCCAGATTGAGCACCACCTCTTCCCCACCATGCCCCGGCACAACTTACAC
AAGATCGCCCCGCTGGTGAAGTCTCTATGTGCCAAGCATGGCATTGAATACCAGGAGAAGCCGCTACTGAGGG
CCCTGCTGGACATCATCAGGTCCCTGAAGAAGTCTGGGAAGCTGTGGCTGGACGCCTACCTTCACAAATGAAG
CCACAGCCCCCGGGACACCGTGGGGAAGGGGTGCAGGTGGGGTGATGGCCAGAGGAATGATGGGCTTTTGTTC
TGAGGGGTGTCCGAGAGGCTGGTGTATGCACTGCTCACGGACCCCATGTTGGATCTTTCTCCCTTTCTCCTCT
CCTTTTTCTCTTCACATCTCCCCCATAGCACCCTGCCCTCATGGGACCTGCCCTCCCTCAGCCGTCAGCCATC
AGCCATGGCCCTCCCAGTGCCTCCTAGCCCCTTCTTCCAAGGAGCAGAGAGGTGGCCACCGGGGGTGGCTCTG
TCCTACCTCCACTCTCTGCCCCTAAAGATGGGAGGAGACCAGCGGTCCATGGGTCTGGCCTGTGAGTCTCCCC
TTGCAGCCTGGTCACTAGGCATCACCCCCGCTTTGGTTCTTCAGATGCTCTTGGGGTTCATAGGGGCAGGTCC
TAGTCGGGCAGGGCCCCTGACCCTCCCGGCCTGGCTTCACTCTCCCTGACGGCTGCCATTGGTCCACCCTTTC
ATAGAGAGGCCTGCTTTGTTACAAAGCTCGGGTCTCCCTCCTGCAGCTCGGTTAAGTACCCGAGGCCTCTCTT
AAGATGTCCAGGGCCCCAGGCCCGCGGGCACAGCCAGCCCAAACCTTGGGCCCTGGAAGAGTCCTCCACCCCA
TCACTAGAGTGCTCTGACCCTGGGCTTTCACGGGCCCCATTCCACCGCCTCCCCAACTTGAGCCTGTGACCTT
GGGACCAAAGGGGGAGTCCCTCGTCTCTTGTGACTCAGCAGAGGCAGTGGCCACGTTCAGGGAGGGGCCGGCT
GGCCTGGAGGCTCAGCCCACCCTCCAGCTTTTCCTCAGGGTGTCCTGAGGTCCAAGATTCTGGAGCAATCTGA
CCCTTCTCCAAAGGCTCTGTTATCAGCTGGGCAGTGCCAGCCAATCCCTGGCCATTTGGCCCCAGGGGACGTG
GGCCCTG

FIG.6

Contig 2535

GTCTTTTACTTTGGCAATGGCTGGATTCCTACCCTCATCACGGCCTTTGTCCTTGCTACCTCTCAGGCCCAAG
CTGGATGGCTGCAACATGATTATGGCCACCTGTCTGTCTACAGAAAACCCAAGTGGAACCACCTTGTCCACAA
ATTCGTCATTGGCCACTTAAAGGGTGCCTCTGCCAACTGGTGGAATCATCGCCACTTCCAGCACCACGCCAAG
CCTAACATCTTCCACAAGGATCCCGATGTGAACATGCTGCACGTGTTTGTTCTGGGCGAATGGCAGCCCATCG
AGTACGGCAAGAAGAAGCTGAAATACCTGCCCTACAATCACCAGCACGAATACTTCTTCCTGATTGGGCCGCC
GCTGCTCATCCCCATGTATTTCCAGTACCAGATCATCATGACCATGATCGTCCATAAGAACTGGGTGGACCTG
GCCTGGGCCGTCAGCTACTACATCCGGTTCTTCATCACCTACATCCCTTTCTACGGCATCCTGGGAGCCCTCC
TTTTCCTCAACTTCATCAGGTTCCTGGAGAGCCACTGGTTTGTGTGGGTCACACAGATGAATCACATCGTCAT
GGAGATTGACCAGGAGGCCTACCGTGACTGGTTCAGTAGCCAGCTGACAGCCACCTGCAACGTGGAGCAGTCC
TTCTTCAACGACTGGTTCAGTGGACACCTTAACTTCCAGATTGAGCACCACCTCTTCCCCACCATGCCCCGGC
ACAACTTACACAAGATCGCCCCGCTGGTGAAGTCTCTATGTGCCAAGCATGGCATTGAATACCAGGAGAAGCC
GCTACTGAGGGCCCTGCTGGACATCATCAGGTCCCTGAAGAAGTCTGGGAAGCTGTGGCTGGACGCCTACCTT
CACAAATGAAGCCACAGCCCCCGGGACACCGTGGGGAAGGGGTGCAGGTGGGGTGATGGCCAGAGGAATGATG
GGCTTTTGTTCTGAGGGGTGTCCGAGAGGCTGGTGTATGCACTGCTCACGGACCCCATGTTGGATCTTTCTCC
CTTTCTCCTCTCCTTTTTCTCTTCACATCTCCCCCATAGCACCCTGCCCTCATGGGACCTGCCCTCCCTCAGC
CGTCAGCCATCAGCCATGGCCCTCCCAGTGCCTCCTAGCCCCTTCTTCCAAGGAGCAGAGAGGTGGCCACCGG
GGGTGGCTCTGTCCTACCTCCACTCTCTGCCCCTAAAGATGGGAGGAGACCAGCGGTCCATGGGTCTGGCCTG
TGAGTCTCCCCTTGCAGCCTGGTCACTAGGCATCACCCCCGCTTTGGTTCTTCAGATGCTCTTGGGGTTCATA
GGGGCAGGTCCTAGTCGGGCAGGGCCCCTGACCCTCCCGGCCTGGCTTCACTCTCCCTGACGGCTGCCATTGG
TCCACCCTTTCATAGAGAGGCCTGCTTTGTTACAAAGCTCGGGTCTCCCTCCTGCAGCTCGGTTAAGTACCCG
AGGCCTCTCTTAAGATGTCCAGGGCCCCAGGCCCGCGGGCACAGCCAGCCCAAACCTTGGGCCCTGGAAGAGT
CCTCCACCCCATCACTAGAGTGCTCTGACCCTGGGCTTTCACGGGCCCCATTCCACCGCCTCCCCAACTTGAG
CCTGTGACCTTGGGACCAAAGGGGGAGTCCCTCGTCTCTTGTGACTCAGCAGAGGCAGTGGCCACGTTCAGGG
AGGGGCCGGCTGGCCTGGAGGCTCAGCCCACCCTCCAGCTTTTCCTCAGGGTGTCCTGAGGTCCAAGATTCTG
GAGCAATCTGACCCTTCTCCAAAGGCTCTGTTATCAGCTGGGCAGTGCCAGCCAATCCCTGGCCATTTGGCCC
CAGGGGACGTGGGCCCTG

FIG.7

Edited Contig 253538a

CAGGGACCTACCCCGCGCTACTTCACCTGGGACGAGGTGGCCCAGCGCTCAGGGTGCGAGGAGCGGTGGCTAGTGATCGA
CCGTAAGGTGTACAACATCAGCGAGTTCACCCGCCGGCATCCAGGGGGCTCCCGGGTCATCAGCCACTACGCCGGGCAGG
ATGCCACGGATCCCTTTGTGGCCTTCCACATCAACAAGGGCCTTGTGAAGAAGTATATGAACTCTCTCCTGATTGGAGAA
CTGTCTCCAGAGCAGCCCAGCTTTGAGCCCACCAAGAATAAAGAGCTGACAGATGAGTTCCGGGAGCTGCGGGCCACAGT
GGAGCGGATGGGGCTCATGAAGGCCAACCATGTCTTCTTCCTGCTGTACCTGCTGCACATCTTGCTGCTGGATGGTGCAG
CCTGGCTCACCCTTTGGGTCTTTGGGACGTCCTTTTTGCCCTTCCTCCTCTGTGCGGTGCTGCTCAGTGCAGTTCAGCAG
GCCCAAGCTGGATGGCTGCAACATGATTATGGCCACCTGTCTGTCTACAGAAAACCCAAGTGGAACCACCTTGTCCACAA
ATTCGTCATTGGCCACTTAAAGGGTGCCTCTGCCAACTGGTGGAATCATCGCCACTTCCAGCACCACGCCAAGCCTAACA
TCTTCCACAAGGATCCCGATGTGAACATGCTGCACGTGTTTGTTCTGGGCGAATGGCAGCCCATCGAGTACGGCAAGAAG
AAGCTGAAATACCTGCCCTACAATCACCAGCACGAATACTTCTTCCTGATTGGGCCGCCGCTGCTCATCCCCATGTATTT
CGAGTACCAGATCATCATGACCATGATCGTCGATAAGAACTGGGTGGAGCTGGCCTGGGCCGTCAGCTACTACATCCGGT
TCTTCATCACCTACATCCCTTTCTACGGCATCCTGGGAGCCCTCCTTTTCCTCAACTTCATCAGGTTCCTGGAGAGCCAC
TGGTTTGTGTGGGTCACACAGATGAATCACATCGTCATGGAGATTGACCAGGAGGCCTACCGTGACTGGTTCAGTAGCCA
GCTGACAGCCACCTGCAACGTGGAGCAGTCCTTCTTCAACGACTGGTTCAGTGGACACCTTAACTTCCAGATTGAGCACC
ACCTCTTCCCCACCATGCCCCGGCACAACTTACACAAGATCGCCCCGCTGGTGAAGTCTCTATGTGCCAAGCATGGCATT
GAATACCAGGAGAAGCCGCTACTGAGGGCCCTGCTGGACATCATCAGGTCCCTGAAGAAGTCTGGGAAGCTGTGGCTGGA
CGCCTACCTTCACAAATGAAGCCACAGCCCCCGGGACACCGTGGGGAAGGGGTGCAGGTGGGGTGATGGCCAGAGGAATG
ATGGGCTTTTGTTCTGAGGGGTGTCCGAGAGGCTGGTGTATGCACTGCTCACGGACCCCATGTTGGATCTTTCTCCCTTT
CTCCTCTCCTTTTTCTCTTCACATCTCCCCCATAGCACCCTGCCCTCATGGGACCTGCCCTCCCTCAGCCGTCAGCCATC
AGCCATGGCCCTCCCAGTGCCTCCTAGCCCCTTCTTCCAAGGAGCAGAGAGGTGGCCACCGGGGGTGGCTCTGTCCTACC
TCCACTCTCTGCCCCTAAAGATGGGAGGAGACCAGCGGTCCATGGGTCTGGCCTGTGAGTCTCCCCTTGCAGCCTGGTCA
CTAGGCATCACCCCCGCTTTGGTTCTTCAGATGCTCTTGGGGTTCATAGGGGCAGGTCCTAGTCGGGCAGGGCCCCTGAC
CCTCCCGGCCTGGCTTCACTCTCCCTGACGGCTGCCATTGGTCCACCCTTTCATAGAGAGGCCTGCTTTGTTACAAAGCT
CGGGTCTCCCTCCTGCAGCTCGGTTAAGTACCCGAGGCCTCTCTTAAGATGTCCAGGGCCCCAGGCCCGCGGGCACAGCC
AGCCCAAACCTTGGGCCCTGGAAGAGTCCTCCACCCCATCACTAGAGTGCTCTGACCCTGGGCTTTCACGGGCCCCATTC
CACCGCCTCCCCAACTTGAGCCTGTGACCTTGGGACCAAAGGGGGAGTCCCTCGTCTCTTGTGACTCAGCAGAGGCAGTG
GCCACGTTCAGGGAGGGGCCGGCTGGCCTGGAGGCTCAGCCCACCCTCCAGCTTTTCCTCAGGGTGTCCTGAGGTCCAAG
ATTCTGGAGCAATCTGACCCTTCTCCAAAGGCTCTGTTATCAGCTGGGCAGTGCCAGCCAATCCCTGGCCATTTGGCCCC
AGGGGACGTGGGCCCTG

FIG.8

FastA Match of Ma29 and contig 253538a

```
SCORES      Init1:  117 Initn:  225 Opt:  256
Smith-Waterman score: 408;   27.0% identity in 441 aa overlap

                    10        20        30        40        50
Ma29.pep      MGTDQGKT---FTWEELAAHNTKDDLLLAIRGRVYDVTKFLSRHPGGVDTLLLGAGRDVT
                 || |   |||:|:| ::  ::  |:|  :||::::|  |||||  ::    ||:|:|
253538a           QGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVISHYAGQDAT
                         10        20        30        40        50

               60        70        80        90       100       110
Ma29.pep      PVFEMYHAF-GAADAIMKKYYVGTLVSNELPIFPEPTVFHKTIKTRVEGYFTDRNIDPKN
                |  :|   | :   |::  :| | | | | | |||  ::       |   | : :   :
253538a       DPFVAFHINKGLVKKYMNSLLIGEL-SPEQPSF-EPTKNKELTDEFRELRATVERMGLMK
                60        70        80          90       100       110

              120       130       140       150       160       170
Ma29.pep      RPEIWGRYALIFGSLIASYYAQLFVPFVVERTWLQVVF-AIIMGFACAQVGLNPLHDASH
                :::  : | :  ::     |  :: :|   ::|  :: |:::: : ||:|    || :|
253538a       ANHVF--FLLYLLHILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQ-HDYGH
                   120       130       140       150       160       170

                180       190           200       210       220
Ma29.pep      FSVTHNPTVWKILGATHDF----FNGASYLVWMYQHMLGHHPYTNIAGADPDVSTSE---
              :|| ::|  |: |  :| |    ::|||   | ::| : ||   ||   ||||:  :
253538a       LSVYRKPK-WNHL--VHKFVIGHLKGASANWWNHRH-FQHHAKPNIFHKDPDVNMLHVFV
                     180       190       200       210       220

                230       240       250       260       270       280
Ma29.pep      ----PDVRRIKPNQKWF-VNHINQHMFV--PFLYGLLAFKVRIQDINILYFVKTNDAIRV
                   ::  | : |::  || ::::|:  | |   : |: :|    |: ::  :: : :
253538a       LGEWQPIEYGKKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQI----IMTMIVHKNWVDL
              230       240       250       260       270           280

                    290       300       310       320       330       340
Ma29.pep      NPISTWHTVMFWGGKAFFVWYRLIVPLQYLPLGKVLLLFTVADMVSSYWLALTFQANHVV
                 :| : ::     ||: |   :|: |  || :||:::   :: |:|:: : | ||:|
253538a       ----AWAVSYYI---RFFITY---IPF-YGILG-ALLFLNFIRFLESHWFVWVTQMNHIV
                        290           300       310       320       330

                    350       360           370       380       390
Ma29.pep      EEVQWPLPDENGIIQKDWAAMQVETT----QDYAHDSHLWTSITGSLNYQAVHHLFPNVS
               |:     |:::   :|| : |: :|    |:: :|   | |  | ||:|  |||||::
253538a       MEI-----DQEAY--RDWFSSQLTATCNVEQSFFND---WFS--GHLNFQIEHHLFPTMP
                         340       350       360           370
```

FIG.9A

```
                400         410         420         430         440
Ma29.pep        QHHYPDILAIIKNTCSEYKVPYLVKDTFWQAFASHLEHLRVLGLRPKEEX
                :|:    |   ::|: |::: : |   |
253538a         RHNLHKIAPLVKSLCAKHGIEYQEKPLLRALLDIIRSLKKSGKLWLDAYLHKX
              380         390         400         410         420         430
```

FIG.9B

FastA Match of Ma524 and contig 253538a

SCORES Init1: 231 Initn: 499 Opt: 401
Smith-Waterman score: 620; 27.3% identity in 455 aa overlap

```
                10        20        30        40        50        59
Ma524.pep     MAAAPSVRTFTRAEVLNAEALNEGKKDAEAPFLMIIDNKVYDVREFVPDHPGGSVILTH-
                |: | ||  ||        :::::     ::|| |||:: ||:  ||||| :::|
253538a         QGPTPRYFTWDEV--------AQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVISHY
                       10                20        30        40        50

            60        70          80        90          100       110
Ma524.pep     VGKDGTDVFDTFHPEAAW--ETLANFYVGDIDE---SDRDIKNDDFAAEVRKLRTLFQSL
              :|:|:|| | :|| : :   : : :: :|:::    | :  || ::: | |:||:  : :
253538a       AGQDATDPFVAFHINKGLVKKYMNSLLIGELSPEQPSFEPTKNKELTDEFRELRATVERM
                     60        70        80        90        100       110

               120       130       140       150       160       170
Ma524.pep     GYYDSSKAYYAFKVSFNLCIWGLSTVIVAKWGQTSTLANVLSAALLGLFWQQCGWLAHDF
              | : :::::: : :- | :-| : : :  :| || |  :| |:||:    | ||| ||:
253538a       GLMKANHVFFLLYLLHILLLDGAAWLTLWVFG-TSFLPFLLCAVLLSAVQAQAGWLQHDY
                    120       130       140       150       160

               180       190       200       210       220       230
Ma524.pep     LHHQVFQDRFWGDLFGAFLGGVCQGFSSSWWKDKHNTHHAAPNVHGEDPDIDTHPLLTWS
               | :|::   |: |   |: |  :| |::||: :|  ||| ||:  :|||::   :|
253538a       GHLSVYRKPKWNHLVHKFVIGHLKGASANWWNHRHFQHHAKPNIFHKDPDVN---ML---
            170       180       190       200       210       220

               240       250       260       270          280       290
Ma524.pep     EHALEMFSDVPDEELTRMWSRFMVLNQTWFYFPILS---FARLSWCLQSILFVLPNGQAH
               |:: ::::    |  :   :::  |:   || :::   :  : :  | |: ::    :|
253538a       -HVF-VLGEWQPIEYGKKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIIMTMI----VH
                     230       240       250       260       270

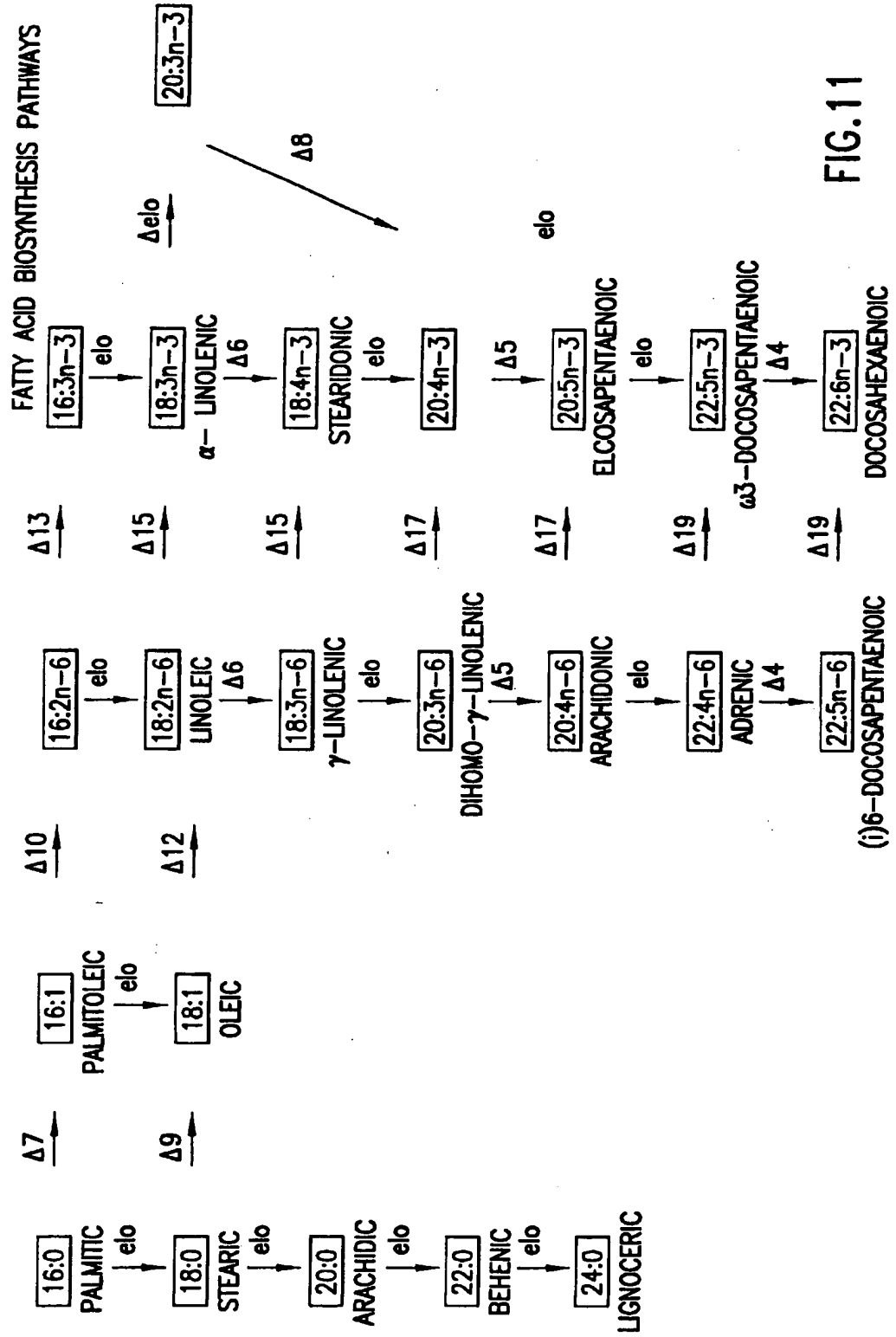
                300       310       320       330       340       349
Ma524.pep     KPSGARVPISLVEQLSLAMHWTWYLATMFLFIK--DPVNMLVYFLVSQAVCGNLLAIVFS
              |          : ::||   ::|:  :: :|     :: |:::   : : :: :: | :
253538a       K----------NWVDLAWAVSYYIRFFITYIPFYGILGALLFLNFIRFLESHWFVWVTQ
                           280       290       300       310       320

            350       360       370       380       390       400       409
Ma524.pep     LNHNGMPVISKEEAVDMDFFTKQIITGRDVHPGLFANWFTGGLNYQIEHHLFPSMPRHNF
              :||  | :   :||   |:|::|: :  :|: ::| :||:| ||:|||||||:|||||:
253538a       MNHIVMEI--DQEAYR-DWFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHLFPTMPRHNL
               330       340       350       360       370       380
```

FIG.10A

```
                410        420        430        440        450
Ma524.pep     SKIQPAVETLCKKYNVRYHTTGMIEGTAEVFSRLNEVSKAASKMGKAQX
                || | |::|| |::::|:    ::::  :::   |:: :|
253538a       HKIAPLVKSLCAKHGIEYQEKPLLRALLDIIRSLKKSGKLWLDAYLHKX
                   390        400        410        420        430
```

FIG.10B


FATTY ACID BIOSYNTHESIS PATHWAYS
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PALMITIC
elo
18:0
STEARIC
elo
20:0
ARACHIDIC
elo
22:0
BEHENIC
elo
24:0
LIGNOCERIC
Δ7
Δ9
16:1
PALMITOLEIC
elo
18:1
OLEIC
Δ10
Δ12
16:2n−6
elo
18:2n−6
LINOLEIC
Δ6
18:3n−6
γ−LINOLENIC
elo
20:3n−6
DIHOMO−γ−LINOLENIC
Δ5
20:4n−6
ARACHIDONIC
elo
22:4n−6
ADRENIC
Δ4
22:5n−6
(i)6−DOCOSAPENTAENOIC
Δ13
Δ15
16:3n−3
elo
18:3n−3
α− LINOLENIC
Δ6
18:4n−3
STEARIDONIC
elo
20:4n−3
Δ5
20:5n−3
ELCOSAPENTAENOIC
elo
22:5n−3
ω3−DOCOSAPENTAENOIC
Δ4
22:6n−3
DOCOSAHEXAENOIC
Δ15
Δ17
Δ17
Δ19
Δ19
Δelo
20:3n−3
Δ8
elo

FIG. 11

Human D5-desaturase

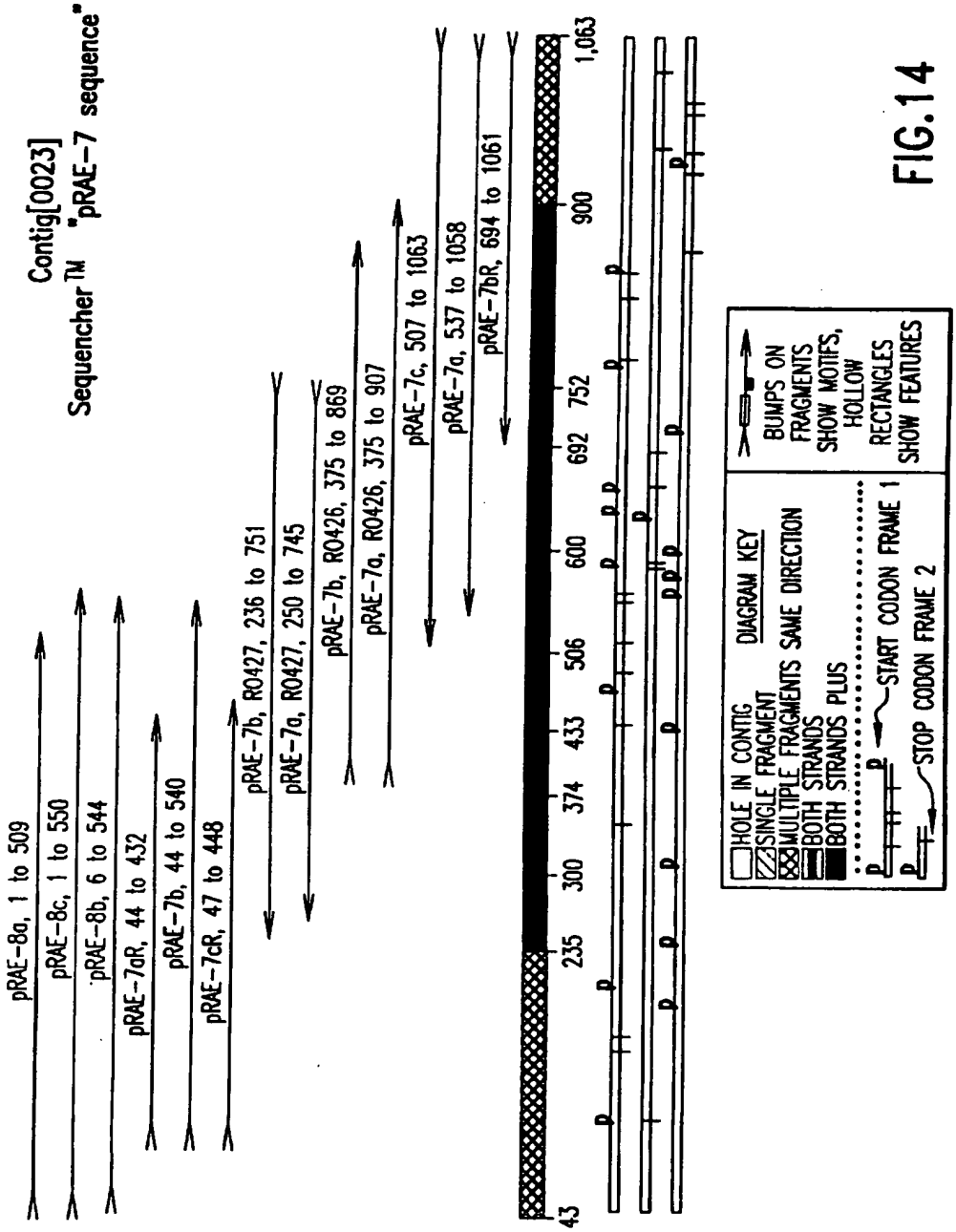
ATGGCCCCCGACCCGGTGGCCGCCGAGACCGCGGCTCAGGGACCTACCCCGCGCTACTTCACCTGGGACGAGG
TGGCCCAGCGCTCAGGGTGCGAGGAGCGGTGGCTAGTGATCGACCGTAAGGTGTACAACATCAGCGAGTTCAC
CCGCCGGCATCCAGGGGGCTCCCGGGTCATCAGCCACTACGCCGGGCAGGATGCCACGGATCCCTTTGTGGCC
TTCCACATCAACAAGGGCCTTGTGAAGAAGTATATGAACTCTCTCCTGATTGGAGAACTGTCTCCAGAGCAGC
CCAGCTTTGAGCCCACCAAGAATAAAGAGCTGACAGATGAGTTCCGGGAGCTGCGGGCCACAGTGGAGCGGAT
GGGGCTCATGAAGGCCAACCATGTCTTCTTCCTGCTGTACCTGCTGCACATCTTGCTGCTGGATGGTGCAGCC
TGGCTCACCCTTTGGGTCTTTGGGACGTCCTTTTTGCCCTTCCTCCTCTGTGCGGTGCTGCTCAGTGCAGTTC
AGGCCCAGGCTGGCTGGCTGCAGCATGACTTTGGGCACCTGTCGGTCTTCAGCACCTCAAAGTGGAACCATCT
GCTACATCATTTTGTGATTGGCCACCTGAAGGGGGCCCCCGCCAGTTGGTGGAACCACATGCACTTCCAGCAC
CATGCCAAGCCCAACTGCTTCCGCAAAGACCCAGACATCAACATGCATCCCTTCTTCTTTGCCTTGGGGAAGA
TCCTCTCTGTGGAGCTTGGGAAACAGAAGAAAAAATATATGCCGTACAACCACCAGCACAAATACTTCTTCCT
AATTGGGCCCCCAGCCTTGCTGCCTCTCTACTTCCAGTGGTATATTTTCTATTTTGTTATCCAGCGAAAGAAG
TGGGTGGACTTGGCCTGGATGATTACCTTCTACGTCCGCTTCTTCCTCACTTATGTGCCACTATTGGGGCTGA
AAGCCTTCCTGGGCCTTTTCTTCATAGTCAGGTTCCTGGAAAGCAACTGGTTTGTGTGGGTGACACAGATGAA
CCATATTCCCATGCACATTGATCATGACCGGAACATGGACTGGGTTTCCACCCAGCTCCTGGCCACATGCAAT
GTCCACAAGTCTGCCTTCAATGACTGGTTCAGTGGACACCTCAACTTCCAGATTGAGCACCATCTTTTTCCCA
CGATGCCTCGACACAATTACCACAAAGTGGCTCCCCTGGTGCAGTCCTTGTGTGCCAAGCGTGGCATAGAGTA
CCAGTCCAAGCCCCTGCTGTCAGCCTTCGCCGACATCATCCACTCACTAAAGGAGTCAGGGCAGCTCTGGCTA
GATGCCTATCTTCACCAATAA

FIG.12

Human D5-desaturase

```
  1  MAPDPVAAET AAQGPTPRYF TWDEVAQRSG CEERWLVIDR KVYNISEFTR
 51  RHPGGSRVIS HYAGQDATDP FVAFHINKGL VKKYMNSLLI GELSPEQPSF
101  EPTKNKELTD EFRELRATVE RMGLMKANHV FFLLYLLHIL LLDGAAWLTL
151  WVFGTSFLPF LLCAVLLSAV QAQAGWLQHD FGHLSVFSTS KWNHLLHHFV
201  IGHLKGAPAS WWNHMHFQHH AKPNCFRKDP DINMHPFFFA LGKILSVELG
251  KQKKKYMPYN HQHKYFFLIG PPALLPLYFQ WYIFYFVIQR KKWVDLAWMI
301  TFYVRFFLTY VPLLGLKAFL GLFFIVRFLE SNWFVWVTQM NHIPMHIDHD
351  RNMDWVSTQL LATCNVHKSA FNDWFSGHLN FQIEHHLFPT MPRHNYHKVA
401  PLVQSLCAKR GIEYQSKPLL SAFADIIHSL KESGQLWLDA YLHQ*
```

FIG.13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pRAE-8b, 6 to 544
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pRAE-7b, 44 to 540
pRAE-7cR, 47 to 448
pRAE-7b, RO427, 236 to 751
pRAE-7a, RO427, 250 to 745
pRAE-7b, RO426, 375 to 869
pRAE-7a, RO426, 375 to 907
pRAE-7c, 507 to 1063
pRAE-7a, 537 to 1058
pRAE-7bR, 694 to 1061
43
235
300
374
433
506
600
692
752
900
1,063
DIAGRAM KEY
HOLE IN CONTIG
SINGLE FRAGMENT
MULTIPLE FRAGMENTS SAME DIRECTION
BOTH STRANDS
BOTH STRANDS PLUS
START CODON FRAME 1
STOP CODON FRAME 2
BUMPS ON FRAGMENTS SHOW MOTIFS, HOLLOW RECTANGLES SHOW FEATURES

FIG.14

pRAE-7 Complete Sequence

```
          10          20          30          40
     *    *    *    *    *    *    *    *    *
CTC CTG GAG CCC GTC AGT ATC GGC GGA ATT CCG GCA GTT CAG GCC CAG
Leu Leu Glu Pro Val Ser Ile Gly Gly Ile Pro Ala Val Gln Ala Gln>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

50          60          70          80          90
 *    *    *    *    *    *    *    *    *    *
GCT GGC TGG CTG CAG CAT GAC TTT GGG CAC CTG TCG GTC TTC AGC ACC
Ala Gly Trp Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

  100         110         120         130         140
    *    *    *    *    *    *    *    *    *
TCA AAG TGG AAC CAT CTG CTA CAT CAT TTT GTG ATT GGC CAC CTG AAG
Ser Lys Trp Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

    150         160         170         180         190
*    *    *    *    *    *    *    *    *    *
GGG GCC CCC GCC AGT TGG TGG AAC CAC ATG CAC TTC CAG CAC CAT GCC
Gly Ala Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

      200         210         220         230         240
  *    *    *    *    *    *    *    *    *    *
AAG CCC AAC TGC TTC CGC AAA GAC CCA GAC ATC AAC ATG CAT CCC TTC
Lys Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

          250         260         270         280
     *    *    *    *    *    *    *    *    *
TTC TTT GCC TTG GGG AAG ATC CTC TCT GTG GAG CTT GGG AAA CAG AAG
Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

290         300         310         320         330
 *    *    *    *    *    *    *    *    *    *
AAA AAA TAT ATG CCG TAC AAC CAC CAG CAC AAA TAC TTC TTC CTA ATT
Lys Lys Tyr Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>
```

FIG.15A

```
  340         350         360         370         380
    *   *   *   *   *   *   *   *   *   *
GGG CCC CCA GCC TTG CTG CCT CTC TAC TTC CAG TGG TAT ATT TTC TAT
Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

    390         400         410         420         430
*   *   *   *   *   *   *   *   *   *
TTT GTT ATC CAG CGA AAG AAG TGG GTG GAC TTG GCC TGG ATG ATT ACC
Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

      440         450         460         470         480
  *   *   *   *   *   *   *   *   *   *
TTC TAC GTC CGC TTC TTC CTC ACT TAT GTG CCA CTA TTG GGG CTG AAA
Phe Tyr Val Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

        490         500         510         520
    *   *   *   *   *   *   *   *   *
GCC TTC CTG GGC CTT TTC TTC ATA GTC AGG TTC CTG GAA AGC AAC TGG
Ala Phe Leu Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

530         540         550         560         570
  *   *   *   *   *   *   *   *   *   *
TTT GTG TGG GTG ACA CAG ATG AAC CAT ATT CCC ATG CAC ATT GAT CAT
Phe Val Trp Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

  580         590         600         610         620
    *   *   *   *   *   *   *   *   *   *
GAC CGG AAC ATG GAC TGG GTT TCC ACC CAG CTC CAG GCC ACA TGC AAT
Asp Arg Asn Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

    630         640         650         660         670
*   *   *   *   *   *   *   *   *   *
GTC CAC AAG TCT GCC TTC AAT GAC TGG TTC AGT GGA CAC CTC AAC TTC
Val His Lys Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

      680         690         700         710         720
  *   *   *   *   *   *   *   *   *   *
CAG ATT GAG CAC CAT CTT TTT CCC ACG ATG CCT CGA CAC AAT TAC CAC
Gln Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>
```

FIG.15B

```
        730         740         750         760
     *     *     *     *     *     *     *     *     *
AAA GTG GCT CCC CTG GTG CAG TCC TTG TGT GCC AAG CAT GGC ATA GAG
Lys Val Ala Pro Leu Val Gln Ser Leu Cys Ala Lys His Gly Ile Glu>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

770         780         790         800         810
  *     *     *     *     *     *     *     *     *     *
TAC CAG TCC AAG CCC CTG CTG TCA GCC TTC GCC GAC ATC ATC CAC TCA
Tyr Gln Ser Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>

  820         830         840         850         860
     *     *     *     *     *     *     *     *     *
CTA AAG GAG TCA GGG CAG CTC TGG CTA GAT GCC TAT CTT CAC CAA TAA
Leu Lys Glu Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln ***>
___a___a___a___a_TRANSLATION OF PRAE-7 MV [A]__a___a___a___a___>
```

FIG.15C

FastA Match of the Gene in pRAE-7 and the M. alpina
D5-desaturase (Ma29) Translated Sequences

```
SCORES      Init1:    62 Initn:   105 Opt:   245
Smith-Waterman score: 271;    28.4% identity in 292 aa overlap

                                        10        20        30        40
pRAE-7.pep                      LLEPVSIGGIPAVQAQAGWLQ-HDFGHLSV-FSTSKWNHL--LH
                                          ||:|    || :|:||  : : |: |   |
Ma29.pep      ASYYAQLFVPFVVERTWLQVVFAIIMGFACAQVGLNPLHDASHFSVTHNPTVWKILGATH
                   140       150       160       170       180       190

                    50        60        70        80        90
pRAE-7.pep    HFVIGHLKGAPASWWNHMH-FQHHAKPNCFRKDPDINM-HPFFFALGKILSVELGKQKKK
                |    ::||     | ::| : ||    |     |||::  :|          :|:  | ::|
Ma29.pep      DF----FNGASYLVWMYQHMLGHHPYTNIAGADPDVSTSEP---------DVRRIKPNQK
                       200       210       220                 230

              100       110       120       130       140       149
pRAE-7.pep    YMPYNH--QHKYF-FLIGPPALLPLYFQWYIFYFVIQ----RKKWVDLAWMITFY--VRF
              ::  ||  || :  || |  |:     :  |:|||      | : ::    : |:    |
Ma29.pep      WF-VNHINQHMFVPFLYGLLAFKVRIQDINILYFVKTNDAIRVNPISTWHTVMFWGGKAF
             240       250       260       270       280       290

             150       160       170       180       190
pRAE-7.pep    FLTY---VPL--LGLKAFLGLFFIVRFLESNWFVWVTQMNHIPMHID---HDRN----MD
              |: |   |||  | |   | || :: :: | |:: : | ||:  :::    |:|     |
Ma29.pep      FVWYRLIVPLQYLPLGKVLLLFTVADMVSSYWLALTFQANHVVEEVQWPLPDENGIIQKD
             300       310       320       330       340       350

              200       210       220       230       240       250
pRAE-7.pep    WVSTQLQATCN-VHKSAFNDWFSGHLNFQIEHHLFPTMPRHNYHKVAPLVQSLCAKHGIE
              |:: |:::| : :| | :   ::| ||:|  |||||:: :|:|  :  :::: |::: :
Ma29.pep      WAAMQVETTQDYAHDSHLWTSITGSLNYQAVHHLFPNVSQHHYPDILAIIKNTCSEYKVP
             360       370       380       390       400       410

              260       270       280
pRAE-7.pep    YQSKPLL-SAFADIIHSLKESGQLWLDAYLHQX
              |  |  : :|||: :: |:  |
Ma29.pep      YLVKDTFWQAFASHLEHLRVLGLRPKEEX
             420       430       440
```

FIG.16

FastA Match of the Gene in pRAE-7 and M. alpina
D6-desaturase (Ma524) Translated Sequences

SCORES Init1: 278 Initn: 483 Opt: 301
Smith-Waterman score: 498; 31.9% identity in 285 aa overlap

```
                                    10        20        30        40
pRAE-7.pep                    LLEPVSIGGIPAVQAQAGWLQHDFGHLSVFSTSKWNHLLHHFVIG
                                                                    
Ma524.pep     GLSTVIVAKWGQTSTLANVLSAALLGLFWQQCGWLAHDFLHHQVFQDRFWGDLFGAFLGG
                140       150       160       170       180       190

                 50        60        70        80            90
pRAE-7.pep    HLKGAPASWWNHMHFQHHAKPNCFRKDPDINMHPFF----FALGKILSV---ELGKQKKK
                                                                    
Ma524.pep     VCQGFSSSWWKDKHNTHHAAPNVHGEDPDIDTHPLLTWSEHALEMFSDVPDEELTRMWSR
                200       210       220       230       240       250

              100       110       120       130                   140
pRAE-7.pep    YMPYNHQHKYFFLIGPPALLPLYFQWYIFYFV------------IQRKKWVDLAWMITF
                                                                    
Ma524.pep     FMVLN-QTWFYFPILSFARLSWCLQSILFVLPNGQAHKPSGARVPISLVEQLSLAMHWTW
                260       270       280       290       300       310

                150       160       170       180       190       200
pRAE-7.pep    YVRFFLTYV--PLLGLKAFLGLFFIVRFLESNWFVWVTQMNHIPMHI---DHDRNMDWVS
                                                                    
Ma524.pep     YLATMFLFIKDPV----NMLVYFLVSQAVCGNLLAIVFSLNHNGMPVISKEEAVDMDFFT
                320       330       340       350       360       370

                      210       220       230       240       250       260
pRAE-7.pep    TQLQATCNVHKSAFNDWFSGHLNFQIEHHLFPTMPRHNYHKVAPLVQSLCAKHGIEYQSK
                                                                    
Ma524.pep     KQIITGRDVHPGLFANWFTGGLNYQIEHHLFPSMPRHNFSKIQPAVETLCKKYNVRYHTT
                      380       390       400       410       420       430

                      270       280
pRAE-7.pep    PLLSAFADIIHSLKESGQLWLDAYLHQX
                                    
Ma524.pep     GMIEGTAEVFSRLNEVSKAASKMGKAQX
                      440       450
```

FIG.17

FastA Match of the Gene in pRAE-7 and contig 2535

SCORES Init1: 1028 Initn: 1424 Opt: 1430
Smith-Waterman score: 1430; 71.0% identity in 276 aa overlap

```
                          10        20        30        40        50
pRAE-7.pep          LLEPVSIGGIPAVQAQAGWLQHDFGHLSVFSTSKWNHLLHHFVIGHLKGAPA
                                  ||||||||||:|||||: . |||||:|:|||||||||  |
2535          VFYFGNGWIPTLITAFVLATSQAQAGWLQHDYGHLSVYRKPKWNHLVHKFVIGHLKGASA
                     10        20        30        40        50        60

                60        70        80        90       100       110
pRAE-7.pep    SWWNHMHFQHHAKPNCFRKDPDINM-HPFFFALGKILSVELGKQKKKYMPYNHQHKYFFL
              :|||| |||||||||  |:|||||:|| |   |:||:   :| ||:| ||:|||||||:||||
2535          NWWNHRHFQHHAKPNIFHKDPDVNMLH--VFVLGEWQPIEYGKKKLKYLPYNHQHEYFFL
                     70        80        90       100       110

               120       130       140       150       160       170
pRAE-7.pep    IGPPALLPLYFQWYIFYFVIQRKKWVDLAWMITFYVRFFLTYVPLLG-LKAFLGLFFIVR
              |||| |:|:|||: |:: :| :|:|||||| :::|:|||:||:|: | | |:| | || |
2535          IGPPLLIPMYFQYQIIMTMIVHKNWVDLAWAVSYYIRFFITYIPFYGILGALLFLNFI-R
             120       130       140       150       160       170

               180       190       200       210       220       230
pRAE-7.pep    FLESNWFVWVTQMNHIPMHIDHDRNMDWVSTQLQATCNVHKSAFNDWFSGHLNFQIEHHL
              ||||:|||||||||||| |:||::   || |:|| |||||::| ||||||||||||||||
2535          FLESHWFVWVTQMNHIVMEIDQEAYRDWFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHL
              180       190       200       210       220       230

               240       250       260       270       280
pRAE-7.pep    FPTMPRHNYHKVAPLVQSLCAKHGIEYQSKPLLSAFADIIHSLKESGQLWLDAYLHQX
              |||||||| ||:|||||:|||||||||||| |||| |: |||:|||:||:|||||||:|
2535          FPTMPRHNLHKIAPLVKSLCAKHGIEYQEKPLLRALLDIIRSLKKSGKLWLDAYLHKXSH
              240       250       260       270       280       290

2535          SPRDTVGKGCRWGDGQRNDGLLFXGVSERLVYALLTDPMLDLSPFLLSFFSSHLPHSTLP
              300       310       320       330       340       350
```

FIG.18

FastA Match of the Gene in pRAE-7 and contig 38

```
SCORES      Init1:   965 Initn:   965 Opt:   968
Smith-Waterman score: 968;    97.0% identity in 133 aa overlap

                                          10        20        30        39
pRAE-7.pep                          LLEPVSIGGIPAVQAQAGWLQHDFGHLSVFSTSKWNHLL
                                            :||||||||||||||||||||||||||||
38          LHILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQHDFGHLSVFSTSKWNHLL
                  130       140       150       160       170       180

           40        50        60        70        80        90        99
pRAE-7.pep  HHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPDINMHPFFFALGKILSVELGKQKKKY
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38          HHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPDINMHPFFFALGKILSVELGKQKKKY
               190       200       210       220       230       240

          100       110       120       130       140       150       159
pRAE-7.pep  MPYNHQHKYFFLIGPPALLPLYFQWYIFYFVIQRKKWVDLAWMITFYVRFFLTYVPLLGL
            |||||||:||||||||||||||||||||||||||||||||||||||||||||||||||||
38          MPYNHQHXYFFLIGPPALLPLYFQWYIFYFVIQRKKWVDLAWISKQEYDEAGLPLSTANA
               250       260       270       280       290       300

          160       170       180       190       200       210       219
pRAE-7.pep  KAFLGLFFIVRFLESNWFVWVTQMNHIPMHIDHDRNMDWVSTQLQATCNVHKSAFNDWFS

38          SK
```

FIG.19

FastA Match of the N-terminus of Clone A-1 and Human Cytochrome b5

```
A-1.pdt
SW:CYB5_HUMAN

ID   CYB5_HUMAN     STANDARD;      PRT:   133 AA.
AC   P00167;
DT   21-JUL-1986 (REL. 01, CREATED)
DT   01-NOV-1988 (REL. 09, LAST SEQUENCE UPDATE)
DT   01-FEB-1996 (REL. 33, LAST ANNOTATION UPDATE)
DE   CYTOCHROME B5. . . .


SCORES      Init1:   127  Initn:   127  Opt:   183 z-score: 226.9 E(): 5.4e-06
Smith-Waterman score: 183;    32.2% identity in 90 aa overlap

                530       540       550       560       570       580
A-1.pdt         XLDLPTNMMEXRKAAAELXAAETAAQGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISE
                                                :|:| :|: ::: : ||:: :|||::::
CYB5_HUMAN                                AEQSDEAVKYYTLEEIQKHNHSKSTWLILHHKVYDLTK
                                                10        20        30

                590       600       610       620       630       640
A-1.pdt         FTRRHPGGSRVISHYAGQDATDPFVAFHINKGLVKKYMN-SLLIGELSPEQPSFEPTKNK
                | ::|||| :|: : || |||: |    ::::   : |: :::|||| |::   :|  ||
CYB5_HUMAN      FLEEHPGGEEVLREQAGGDATENFE--DVGHSTDAREMSKTFIIGELHPDD---RPKLNK
                40        50        60        70        80              90

                650       660       670       680       690       700
A-1.pdt         ELTDEFRELRATVEQRFPVXFLTCTGAHGFFSLEVPGLPDSNKXFSWTSRPIXWNKGKRP

CYB5_HUMAN      PPETLITTIDSSSSWWTNWVIPAISAVAVALMYRLYMAED
                    100       110       120       130
```

FIG.20

FastA Match of 5' Sequence of Clone A-1 and ac004228

```
LOCUS       AC004228   170743 bp    DNA             HTG       26-FEB-1998
DEFINITION  *** SEQUENCING IN PROGRESS *** Homo sapiens Chromosome 11q12 pac
            pDJ519o3; HTGS phase 1, 18 unordered pieces.
ACCESSION   AC004228
NID         g2911733
KEYWORDS    HTG; HTGS_PHASE1. . . .

SCORES      Init1:   913 Initn:  1123 Opt:   916
  94.6% identity in 203 bp overlap

            389       379       369       359       349       339       330
A-1           CCCGACCAATATGATGGAATAAAGGAAAGCGGCCGCTGAATTATAGGCCGCCGAGACCGC
                                                  |||| | ||     |||||||||||||||
ac004228      CCCGGCGCGCGGCGTCGCCAGGCCAGCTATGGCCCCCGACCCGGTGGCCGCCGAGACCGC
            60090     60100     60110     60120     60130     60140

            329       319       309       299       289       279       270
A-1           GGCTCAGGGACCTACCCCGCGTTACTTCACATGGGACGAGGTGGCCCAGCGCTCAGGGTG
              |||||||||||||||||||||| |||||||| ||||||||||||||||||||||||||||||
ac004228      GGCTCAGGGACCTACCCCGCGCTACTTCACCTGGGACGAGGTGGCCCAGCGCTCAGGGTG
            60150     60160     60170     60180     60190     60200

            269       259       249       239       229       219       210
A-1           CGAGGAGCGGTGGCTTGTGATCGACCGTAAGGTGTACAACATCAGCGAGTTCACCCGCCG
              |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
ac004228      CGAGGAGCGGTGGCTAGTGATCGACCGTAAGGTGTACAACATCAGCGAGTTCACCCGCCG
            60210     60220     60230     60240     60250     60260

            209       199       189       179       169       159       150
A-1           GCATCCAGGGGGCTCCCGGGTCATCAGCCACTACGCCGGGCAGGATGCCACGGATCCCTT
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228      GCATCCAGGGGGCTCCCGGGTCATCAGCCACTACGCCGGGCAGGATGCCACGGTGAGCGC
            60270     60280     60290     60300     60310     60320

            149       139       129       119       109       99        90
A-1           CGTGGCCTTCCACATCAACAAGGGCCTTGTGAAGAAGTATATGAACTCTCTCCTGATTGG

ac004228      AGCCAGGCGGGGGCACAGGAGAGGGCGGGACCGGAGGCTGAGTGCAGGGGAGACAGAGTT
            60330     60340     60350     60360     60370     60380
```

FIG.21

FastA Match of 5' Sequence of Clone 3-5 and ac004228

SCORES Init1: 1365 Initn: 2510 Opt: 1377
98.6% identity in 285 bp overlap

```
                20        30        40        50        60        70
3-5             AATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGTCCGGACCTGCCAACGTGA
                                                 |||||||   | ||||||||||||||||||
ac004228        CCCCGCCCCACACGCCGCATCACTTACAGGGCCCGGGGCTG-CCGGACCTGCCAACGTGA
                  61710     61720     61730     61740     61750     61760

                  80        90       100       110       120       130
3-5             ATCTTATCGCCATGGACCTTACCTTGCACAACCCAAAGTAGCTGCCTTGGGGCAGGGGGT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228        ATCTTATCGCCATGGACCTTACCTTGCACAACCCAAAGTAGCTGCCTTGGGGCAGGGGGT
                  61770     61780     61790     61800     61810     61820

                 140       150       160       170       180       190
3-5             GGCCAGAGTGCTTAGGGAAATGTGGAGCCCTACCCAGAACAACGGTGGAGGGAAAGGGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228        GGCCAGAGTGCTTAGGGAAATGTGGAGCCCTACCCAGAACAACGGTGGAGGGAAAGGGAA
                  61830     61840     61850     61860     61870     61880

                 200       210       220       230       240       250
3-5             GAAACGCAGAAGTGCCCCAGTTCGGACGTAGGGAAGTCTTCCTCTTCGTGGTTTTTGGAG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228        GAAACGCAGAAGTGCCCCAGTTCGGACGTAGGGAAGTCTTCCTCTTCGTGGTTTTTGGAG
                  61890     61900     61910     61920     61930     61940

                 260       270       280       290       300       310
3-5             AACCCTAGCTAAGAGAGGAAAGGGACTTATTGAAAGACCCGCAAGAAGGGACGGAAGTCT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228        AACCCTAGCTAAGAGAGGAAAGGGACTTATTGAAAGACCCGCAAGAAGGGACGGAAGTCT
                  61950     61960     61970     61980     61990     62000

                 320       330       340       350       360       370
3-5             CATAGCCCTGAGAGGATCCCTTTGTGGCCTTCCACATCAACAAGGGCCTTGTGAAGAAGT
                ||||||||||||||||
ac004228        CATAGCCCTGAGAGGTGAAGCCAGCTGGAGTTGATGGGTCGAATGGGGACCTAGAGAACT
                  62010     62020     62030     62040     62050     62060
```

FIG.22

FastA Match of 5' Sequence of Clone A-10 and ac004228

SCORES Init1: 931 Initn: 1309 Opt: 934
97.0% identity in 200 bp overlap

```
          30        40        50        60        70        80        89
A-10       TATAGGGCTCGAGCGGCCGCCCGGGCAGGTGCCCGGAGGCGCCTGATCATACCTGTTGCC
                                                                      ||||||||||||||||||||||||||||||||||||||||
ac004228   CGAGCCAAACACCGACTAATTCGGAGGAAAGCCCGGAGGCGCCTGATCATACCTGTTGCC
          60400     60410     60420     60430     60440     60450

          90       100       110       120       130       140       149
A-10       CGGTGATTGGGTGTCCTGCGGATGCGGGATGAAAAGGCGGGAGAGAGGCCTGGAAAAGTG
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
ac004228   CGGTGATTGGGTGTCCTGCGGATGCGGGATGAAAAGGCGGGAGAGAGGCCTGGAGAAGTG
          60460     60470     60480     60490     60500     60510

         150       160       170       180       190       200       209
A-10       GAGTCTGGGGAGTGGGGATGGAGGCCAACAACACGCACACACAAACAAAGGGTCCCGCCT
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228   GAGTCTGGGGAGTGGGGATGGAGGCCAACAACACGCACACACAAACAAAGGGTCCCGCCT
          60520     60530     60540     60550     60560     60570

         210       220       230       240       250       260       269
A-10       CCCTGCCGTGCATTCCATCTGCAGCCCCGAGCCTCAGGATCCCTTTGTGGCCTTCCACAT
           |||||||||||||||||||||||||||||||||||||||| || ||  | ||
ac004228   CCCTGCCGTGCATTCCATCTGCAGCCCCGAGCCTCAGG-TCTCTGGGCGGGGACAGAACC
          60580     60590     60600     60610     60620     60630
```

FIG.23

FastA Match of 5' Sequence of Clone A-16 and ac004228

```
SCORES      Init1:   985 Initn:  1488 Opt:   997
  98.1% identity in 209 bp overlap

                    40        50        60        70        80        90
A-16          CGAGCGGCCGCCCGGGCAGGTCTAGAATTCAGCGGCCGCTGAAGCCGCGTCTGGACCTAG
                                          ||  |||  ||  |  ||||||||||||||||||
ac004228      AGGGAGTCACATCCTGTCTCGATGGCTAGGAGAGGCAGC-GCAGCCGCGTCTGGACCTAG
                   60720     60730     60740     60750     60760

                   100       110       120       130       140       150
A-16          GTGCCGGTCTCCACTCGCCAGCAGGAGCGGAGAGGGAGCAGGAAAGGAGCCCATTCTCGA
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228      GTGCCGGTCTCCACTCGCCAGCAGGAGCGGAGAGGGAGCAGGAAAGGAGCCCATTCTCGA
         60770     60780     60790     60800     60810     60820

                   160       170       180       190       200       210
A-16          GGATGGGGCTGAAACGGGAAGCTTGGGGAGACCGCTGCCTTGGGGACCCCTGCGTCGTGT
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228      GGATGGGGCTGAAACGGGAAGCTTGGGGAGACCGCTGCCTTGGGGACCCCTGCGTCGTGT
         60830     60840     60850     60860     60870     60880

                   220       230       240       250       260       270
A-16          GAAGACTGGAGGACGCGGAAGGGACAGCGCTGGCCGGGGAGGGCAAGCGGCCGCTGGCGA
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228      GAAGACTGGAGGACGCGGAAGGGACAGCGCTGGCCGGGGAGGGCAAGCGGCCGCTGGCGT
         60890     60900     60910     60920     60930     60940

                   280       290       300       310       320       330
A-16          TCCCTTTGTGGCCTTCCACATCAACAAGGGCCTTGTGAAGAAGTATATGAACTCTCTCCT

ac004228      ACATAAGGGATTGGGAATGGCATACACTTAGCGAGGACCCCCAGAGCTGTTCTCGAATCG
         60950     60960     60970     60980     60990     61000
```

FIG.24

FastA Match of 5' Sequence of Clone A-19 and ac004228

SCORES Initl: 1227 Initn: 1409 Opt: 1532
94.0% identity in 349 bp overlap

```
                60        70        80        90       100       110
A-19        TTATTCCCTTATTTGTCCCTGCCCATGTCCTGCTGATTGGTCCATTTTACCTCTAGCTAG
                                                      ||||||||||||||||||||||||||||||
ac004228    ATAGAGCACTGATTGGTCCATTTTACAGGGTGCTGATTGGTCCATTTTACCTCTAGCTAG
          63250     63260     63270     63280     63290     63300

               120       130       140       150       160       170
A-19        CTAAAGAGCACGGATTGGTGCATTTTGCAAACCTCTGGCTACAGAGGGGTTCTCCAGGTC
            |||||||||||||||||||||||||| ||||||||| |||||||| |||||||| |||
ac004228    CTAAAGAGCACGGATTGGTGCATTTTACAAACCTCTAGCTACAGAAAAGTTCTCCAAGTC
          63310     63320     63330     63340     63350     63360

               180       190       200       210       220       230
A-19        TGCACTCGACCCAGGAAGTCCATCTGGCTTCACCTCTCACTTCAACTTGGGTACAGCCTT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ac004228    TGCACTCGACCCAGGAAGTCCATCTGGCTTCACCTCTCACTTCAACTTGGGTACAGCCTT
          63370     63380     63390     63400     63410     63420

               240       250       260       270       280       290
A-19        CTGGCGGGCAGGAAGATGGCCTTTGGTGCGAACACTGCCGGAGTCCAGGGGGCTGGCTCC
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
ac004228    CTGGCGGGCAGGAGGATGGCCTTTGGTGCGAACACTGCCGGAGTCCAGGGGGCTGGCTCC
          63430     63440     63450     63460     63470     63480

               300       310       320       330       340
A-19        CTCACCTTTCATCTTCTCCCGGCACTTGCAGGATCCCTTTGTGGCC
            |||||||||||||||||||||||||||||||||||||||||||||
ac004228    CTCACCTTTCATCTTCTCCCGGCACTTGCAGGATCCCTTTGTGGCC
          63490     63500     63510     63520     63530
```

FIG.25

Partial Sequence of ac004228

59751 ACTAGAACCG CTGTTCCTAC CGCGGCGCCC CCTGGGAGCC AACGCCGCGA

59801 TGCCCGCCTG ACGTCAGGAA GTCGAATCCG GCGGCGACGC TTTTAGGGAG

59851 CCCGCGAGGG GGCGCGTGTT GGCAGCCCAG CTGTGAGTTG CCCAAGACCC

59901 ACCGGGGGAC GGGATCTCGC TCCCCGCGCC ACGAGGCTCG GCCAATGGGA Possible start

59951 ACGCGCGCTG CGAGGCCCGC CGGTCTGCCC TGCGGTGCTG AAAACCCGGC

60001 GCGCAGGCGG CTGGCTCTGG GCGCGCGCCA GCAAATCCAC TCCTGGAGCC

60051 CGCGGACCCC GAGCACGCGC CTGACAGCCC CTGCTGGCCC GGCGCGCGGC

60101 GTCGCCAGGC CAGCTATGGC CCCCGACCCG GTGGCCGCCG AGACCGCGGC

60151 TCAGGGACCT ACCCCGCGCT ACTTCACCTG GGACGAGGTG GCCCAGCGCT Clone A-1

60201 CAGGGTGCGA GGAGCGGTGG CTAGTGATCG ACCGTAAGGT GTACAACATC

60251 AGCGAGTTCA CCCGCCGGCA TCCAGGGGGC TCCCGGGTCA TCAGCCACTA

60301 CGCCGGGCAG GATGCCACGG TGAGCGCAGC CAGGCGGGGG CACAGGAGAG

60351 GGCGGGACCG GAGGCTGAGT GCAGGGGAGA CAGAGTTACG CACTCCGAGC

60401 CAAACACCGA CTAATTCGGA GGAAAGCCCG GAGGCGCCTG ATCATACCTG

60451 TTGCCCGGTG ATTGGGTGTC CTGCGGATGC GGGATGAAAA GGCGGGAGAG Clone A-10

60501 AGGCCTGGAG AAGTGGAGTC TGGGGAGTGG GGATGGAGGC CAACAACACG

60551 CACACACAAA CAAAGGGTCC CGCCTCCCTG CCGTGCATTC CATCTGCAGC

60601 CCCGAGCCTC AGGTCTCTGG GCGGGGACAG AACCCCGAGC TGGGTAGGCT

60651 AGGAGGGAGG AGAGCAAGGA TGCAGGCCGC CTGGGGAGGG AGGGGGTCAG

60701 TGGCCAGGGG AGGGAGTCAC ATCCTGTCTC GATGGCTAGG AGAGGCAGCG

60751 CAGCCGCGTC TGGACCTAGG TGCCGGTCTC CACTCGCCAG CAGGAGCGGA Clone B-17

60801 GAGGGAGCAG GAAAGGAGCC CATTCTCGAG GATGGGGCTG AAACGGGAAG

60851 CTTGGGGAGA CCGCTGCCTT GGGGACCCCT GCGTCGTGTG AAGACTGGAG

60901 GACGCGGAAG GGACAGCGCT GGCCGGGGAG GGCAAGCGGC CGCTGGCGTA

FIG.26A

60951 CATAAGGGAT TGGGAATGGC ATACACTTAG CGAGGACCCC CAGAGCTGTT

61001 CTCGAATCGC CGGGGAGGCC ACTGAGCCGC AGGCCAGCGA GGTCTTCAGC

61051 TATTCCGCGG AGCGGACCGC TGTTTACGCT CTGGGGCGGT AGGCCCTTCG

61101 CGGGGTCCTG TCCCTTCTTC CCTTGGTCTC ACTGCGGGGT CGGCGCGCGC

61151 CCCAGCCCCA GGCCTGCTGC TTCCCTTTCT AGACCACAGC CCTCAGAGCT

61201 AAGGCCCCGG CGCCTCTCTG CTGGGTTGGA GTCCTGGGGA CTCAGTCCTA

61251 GGGACTCGAA AGTCGGGGCG TTCCCTTCAC CGCGTTTCCC CCTTGGCGGC

61301 CAGAATGGCG TCCCCTCCCC TTGCATCCCC CTCTGATCCC GTGCCCTGCA

61351 GCGTGATGCC CTCCACTGTC CCTATCCACT ACCCTGGCGT CCCAGAGTGT

61401 GCCGCGGGTC ACCAGGTTCC CATAACGTCG CAGCAGAGCT TAGACGCTGC

61451 GGGGCGAAGA CCCGCCCCAC CCTCTGACGC GACCAGCCTA GTGGGCGAGG

61501 CCAGAGCTTG CGCGGGTCAA CCAGAGTGAC CACTCGGGAG CCCTGACTGC

61551 GGCCAAGGGC GCAGGCGTGT CCCGGCGCAT GCGCAGACGA AACAGGCACC

61601 AACGCTGGAG CTTCCCGCAG TGTGATTTGG GGCCGGGGAT GCCGCGGCGG

61651 GGACGGCGAT TGGTCCGTAT GTGTGGTGCC ACCGGCCGCC GCTCCGCCCC

61701 GGCCCCCGCC CCACACGCCG CATCACTTAC AGGGCCCGGG GCTGCCGGAC

61751 CTGCCAACGT GAATCTTATC GCCATGGACC TTACCTTGCA CAACCCAAAG

61801 TAGCTGCCTT GGGGCAGGGG GTGGCCAGAG TGCTTAGGGA AATGTGGAGC Clone 3-5

61851 CCTACCCAGA ACAACGGTGG AGGGAAAGGG AAGAAACGCA GAAGTGCCCC

61901 AGTTCGGACG TAGGGAAGTC TTCCTCTTCG TGGTTTTTGG AGAACCCTAG

61951 CTAAGAGAGG AAAGGGACTT ATTGAAAGAC CCGCAAGAAG GGACGGAAGT

62001 CTCATAGCCC TGAGAGGTGA AGCCAGCTGG AGTTGATGGG TCGAATGGGG

62051 ACCTAGAGAA CTTTTCTGTA TCTAGAGGTT TGTAAAATGC ACCAATCAGT

62101 GCTCTGTAAA AACGCACCAA TTGGCGCTCT GTAGCTAGCT AGAGGTTTGT

62151 AAAATGAGCC AATCAGCAGG ACGTGGGCAG GGACAACTAA GACAATAAAA

62201 GCTGGCCACC CCAGCCAGCT GCTGCAACCC GCTCCAGTTC CCTTACAGGC

FIG.26B

62251 TGTGGAAGCA TTGTTCTTTT GCTCGTCACA CTAAACCTTG CTGCTGCTCA

62301 TTCTTTGGGT CTGCAAAGAG TGTTATTCCT TTAAGAGCTA TAACAGCGGG

62351 AAGGTCCACG GCTCCATTCT TGAAGTCAGT GAGACCATAC CCGCCGGAAG

62401 GAACCAACGC CCGACACAGC CCCACCCATC TCTCCTGTTT CTCACCTATA

62451 CTGAAATTCT TGGGCAAAAG CTGTCTGTGG ACACACCCAG GGGAAAGGCC

62501 AGCCCAGGCA GGTGTTTCTT AGTGGTTCCC CTCAGCCAAT GCTTCCCATT

62551 CCTTGATGCA TCCTTCTAAC TAGAGCAGAT GCTCGGTGAT CTTAAACTGT

62601 GGACACCTGG GAGCACCCTC AAAAGGCAGC TGGGCCTAGG GAGATGGCCT

62651 GTGCTTCTGT GTCAGGAGTT GGTTCCTTCA GGTGGGCTTG TGGTCTCGCT

62701 GACGTCAAGA ATGAAGCCAT GAACCTTCGC GGTGAGTGTT ACAGCTCTTA

62751 CAGGTGGCGT GGACCCAAAG AGTGAGCAGC AGCAAGATTT ATTGTGAAGA

62801 GCAAAGAACA AAGCTTCCAC AGCGTGGAAG GGTACCCGAG CAGGTTGCCG

62851 CTGCTGGACG TTGGGGGGTG TGAGGGGGAG CAGCCTTTTT TTTTCTTTTT

62901 TTTTTGAGAC GGAGTCTCCC TGTCGCCCAG GCTGGAGTGC AGTGGCGCGA

62951 TCTCGGCTCA CTGCAGGCTC CGCCCCCCCC CCGGGGTTCA CGCCATTCTC

63001 CTGCCTCAGC CTCCCGAGTA GCTGGGACTA CAGGCGCCCG CTACCTCGCC

63051 CGGCTAATTT TTTGTATTTT TAGTAGAGAC GGGGTTTCAC TGTGTTAGCC

63101 AGGATGGTCT CGATCTCCTG ACCTCGTGAT CCACCCGCCT TGGCCTCCCA

63151 AAGTGCTGGG ATTACAGGCG TGAGCCACCG CGCCCGGCCG GGAGCAGCTT

63201 TTATTCCCTT ATTTGTCCCT GCCCATGTCC TGCTGATTTG TCCATTTTAT

63251 AGAGCACTGA TTGGTCCATT TTACAGGGTG CTGATTGGTC CATTTTACCT

63301 CTAGCTAGCT AAAGAGCACG GATTGGTGCA TTTTACAAAC CTCTAGCTAC

63351 AGAAAAGTTC TCCAAGTCTG CACTCGACCC AGGAAGTCCA TCTGGCTTCA

63401 CCTCTCACTT CAACTTGGGT ACAGCCTTCT GGCGGGCAGG AGGATGGCCT

63451 TTGGTGCGAA CACTGCCGGA GTCCAGGGGG CTGGCTCCCT CACCTTTCAT Clone B-19

63501 CTTCTCCCGG CACTTGCAGG ATCCCTTTGT GGCCTTCCAC ATCAACAAGG

FIG.26C

63551 GCCTTGTGAA GAAGTATATG AACTCTCTCC TGATTGGAGA ACTGTCTCCA

63601 GAGCAGCCCA GCTTTGAGCC CACCAAGAAT GTAAGACCCT GTGTTTGCTA

63651 TGTCGCAACT ATTGGTTGTT GAGGGGGACA GAGAGGGGGT GGAAGGAGAG

63701 TCTAGATGGA ATCACAGTCA TAGTAATCAC AGTCAGTAGT AGCTCTGGGG

63751 AGTCTTGAGG TCCCTGCTTC TCTTGCATAG TCATGAGGTC ACAGGCCCAA

63801 GGGAGCATGG CTTTGCAACC TATGGCTCCC CCAAGGCTGC CACTACCATG

63851 GCTGCCATCA TTGTTATCAT CATTGTTATC ATATGAGCAC TTACTATGCA

63901 CCAAGCATAA ACTCATAACT CTTACACATT TACAGATGAG ATAACAGGCT

63951 CAGGGAGGTT AAGCAACACA GCCAAGGATC ACACAGTTAG TAAATGGCAG

64001 AGCAAGGACT TAGTCCCCTG AACTCTTAGG CACTATCCCA TGGCACCTCC

64051 TCCTGTCATC CTCATTGTCG TGGTATCTTT GCCTAGGACT GTGGACTTCC

64101 CACAGCTACC TCAGTGGGAG GTCCTTGAGC CTGAGAGGGC CCTTGTCTCC

64151 AGTAGCATTG GGGTGCAGAT GAGAAGAATA ACAGCTCCTC TTCCTCTTCT

64201 GCAGAAAGAG CTGACAGATG AGTTCCGGGA GCTGCGGGCC ACAGTGGAGC

64251 GGATGGGGCT CATGAAGGCC AACCATGTCT TCTTCCTGCT GTACCTGCTG

64301 CACATCTTGC TGCTGGATGG TGCAGCCTGG CTCACCCTTT GGGTCTTTGG

64351 GACGTCCTTT TTGCCCTTCC TCCTCTGTGC GGTGCTGCTC AGTGCAGTTC

64401 AGGTGAGAGC CTTTGGCTTG TCAAGTGCAC AGCAATGCTC AGCATCCCTG

FIG.26D

FastA Match of Human D5-desaturase and Contig 3381584

SCORES Init1: 4480 Initn: 4480 Opt: 4481
99.9% identity in 898 bp overlap

```
                                                  10        20        30
human D5                                          ATGGCCCCCGACCCGGTGGCCGCCGAGACC
                                                  ||||||||||||||||||||||||||||||
3381584     GGCCCGGCGCGCGGCGTCGCCAGGCCAGCTATGGCCCCCGACCCGGTGGCCGCCGAGACC
              80        90        100       110       120       130

                    40        50        60        70        80        90
human D5    GCGGCTCAGGGACCTACCCCGCGCTACTTCACCTGGGACGAGGTGGCCCAGCGCTCAGGG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584     GCGGCTCAGGGACCTACCCCGCGCTACTTCACCTGGGACGAGGTGGCCCAGCGCTCAGGG
              140       150       160       170       180       190

                  100       110       120       130       140       150
human D5    TGCGAGGAGCGGTGGCTAGTGATCGACCGTAAGGTGTACAACATCAGCGAGTTCACCCGC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584     TGCGAGGAGCGGTGGCTAGTGATCGACCGTAAGGTGTACAACATCAGCGAGTTCACCCGC
              200       210       220       230       240       250

                  160       170       180       190       200       210
human D5    CGGCATCCAGGGGGCTCCCGGGTCATCAGCCACTACGCCGGGCAGGATGCCACGGATCCC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584     CGGCATCCAGGGGGCTCCCGGGTCATCAGCCACTACGCCGGGCAGGATGCCACGGATCCC
              260       270       280       290       300       310

                  220       230       240       250       260       270
human D5    TTTGTGGCCTTCCACATCAACAAGGGCCTTGTGAAGAAGTATATGAACTCTCTCCTGATT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584     TTTGTGGCCTTCCACATCAACAAGGGCCTTGTGAAGAAGTATATGAACTCTCTCCTGATT
              320       330       340       350       360       370

                  280       290       300       310       320       330
human D5    GGAGAACTGTCTCCAGAGCAGCCCAGCTTTGAGCCCACCAAGAATAAAGAGCTGACAGAT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584     GGAGAACTGTCTCCAGAGCAGCCCAGCTTTGAGCCCACCAAGAATAAAGAGCTGACAGAT
              380       390       400       410       420       430

                  340       350       360       370       380       390
human D5    GAGTTCCGGGAGCTGCGGGCCACAGTGGAGCGGATGGGGCTCATGAAGGCCAACCATGTC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584     GAGTTCCGGGAGCTGCGGGCCACAGTGGAGCGGATGGGGCTCATGAAGGCCAACCATGTC
              440       450       460       470       480       490
```

FIG.27A

```
                400        410        420        430        440        450
human D5        TTCTTCCTGCTGTACCTGCTGCACATCTTGCTGCTGGATGGTGCAGCCTGGCTCACCCTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584         TTCTTCCTGCTGTACCTGCTGCACATCTTGCTGCTGGATGGTGCAGCCTGGCTCACCCTT
                500        510        520        530        540        550

                460        470        480        490        500        510
human D5        TGGGTCTTTGGGACGTCCTTTTTGCCCTTCCTCCTCTGTGCGGTGCTGCTCAGTGCAGTT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584         TGGGTCTTTGGGACGTCCTTTTTGCCCTTCCTCCTCTGTGCGGTGCTGCTCAGTGCAGTT
                560        570        580        590        600        610

                520        530        540        550        560        570
human D5        CAGGCCCAGGCTGGCTGGCTGCAGCATGACTTTGGGCACCTGTCGGTCTTCAGCACCTCA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584         CAGGCCCAGGCTGGCTGGCTGCAGCATGACTTTGGGCACCTGTCGGTCTTCAGCACCTCA
                620        630        640        650        660        670

                580        590        600        610        620        630
human D5        AAGTGGAACCATCTGCTACATCATTTTGTGATTGGCCACCTGAAGGGGGCCCCCGCCAGT
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584         AAGTGGAACCATCTGCTACATCATTTTGTGATTGGCCACCTGAAGGGGGCCCCCGCCAGT
                680        690        700        710        720        730

                640        650        660        670        680        690
human D5        TGGTGGAACCACATGCACTTCCAGCACCATGCCAAGCCCAACTGCTTCCGCAAAGACCCA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584         TGGTGGAACCACATGCACTTCCAGCACCATGCCAAGCCCAACTGCTTCCGCAAAGACCCA
                740        750        760        770        780        790

                700        710        720        730        740        750
human D5        GACATCAACATGCATCCCTTCTTCTTTGCCTTGGGGAAGATCCTCTCTGTGGAGCTTGGG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584         GACATCAACATGCATCCCTTCTTCTTTGCCTTGGGGAAGATCCTCTCTGTGGAGCTTGGG
                800        810        820        830        840        850

                760        770        780        790        800        810
human D5        AAACAGAAGAAAAAATATATGCCGTACAACCACCAGCACAAATACTTCTTCCTAATTGGG
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584         AAACAGAAGAAAAAATATATGCCGTACAACCACCAGCACAAATACTTCTTCCTAATTGGG
                860        870        880        890        900        910

                820        830        840        850        860        870
human D5        CCCCCAGCCTTGCTGCCTCTCTACTTCCAGTGGTATATTTTCTATTTTGTTATCCAGCGA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3381584         CCCCCAGCCTTGCTGCCTCTCTACTTCCAGTGGTATATTTTCTATTTTGTTATCCAGCGA
                920        930        940        950        960        970
```

FIG.27B

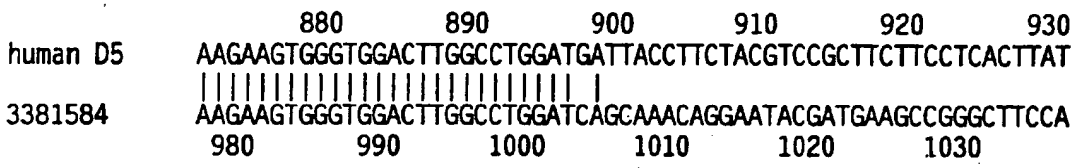
880 890 900 910 920 930
human D5 AAGAAGTGGGTGGACTTGGCCTGGATGATTACCTTCTACGTCCGCTTCTTCCTCACTTAT
3381584 AAGAAGTGGGTGGACTTGGCCTGGATCAGCAAACAGGAATACGATGAAGCCGGGCTTCCA
980 990 1000 1010 1020 1030

FIG.27C

FastA Match of Human D5-desaturase and
Contig 2153526

SCORES Init1: 1892 Initn: 1892 Opt: 2161
98.6% identity in 443 bp overlap

```
                870       880       890       900       910       920
human D5    TCCAGCGAAAGAAGTGGGTGGACTTGGCCTGGATGATTACCTTCTACGTCCGCTTCTTCC
                                                 |  ||::|||||||||||||||||||||||||
2153526                                          GAATKMTTACCTTCTACGTCCGCTTCTTCC
                                                         10        20        30

                930       940       950       960       970       980
human D5    TCACTTATGTGCCACTATTGGGGCTGAAAGCCTTCCTGGGCCTTTTCTTCATAGTCAGGT
            |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
2153526     TCACTTATGTGCCACTATTGGGGCTGAAAG-CTTCCTGGGCCTTTTCTTCATAGTCAGGT
                    40        50        60        70        80

                990       1000      1010      1020      1030      1040
human D5    TCCTGGAAAGCAACTGGTTTGTGTGGGTGACACAGATGAACCATATTCCCATGCACATTG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2153526     TCCTGGAAAGCAACTGGTTTGTGTGGGTGACACAGATGAACCATATTCCCATGCACATTG
          90        100       110       120       130       140

               1050      1060      1070      1080      1090      1100
human D5    ATCATGACCGGAACATGGACTGGGTTTCCACCCAGCTCCTGGCCACATGCAATGTCCACA
            |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
2153526     ATCATGACCGGAACATGGACTGGGTTTCCACCCAGCTCCAGGCCACATGCAATGTCCACA
        150       160       170       180       190       200

               1110      1120      1130      1140      1150      1160
human D5    AGTCTGCCTTCAATGACTGGTTCAGTGGACACCTCAACTTCCAGATTGAGCACCATCTTT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2153526     AGTCTGCCTTCAATGACTGGTTCAGTGGACACCTCAACTTCCAGATTGAGCACCATCTTT
        210       220       230       240       250       260

               1170      1180      1190      1200      1210      1220
human D5    TTCCCACGATGCCTCGACACAATTACCACAAAGTGGCTCCCCTGGTGCAGTCCTTGTGTG
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2153526     TTCCCACGATGCCTCGACACAATTACCACAAAGTGGCTCCCCTGGTGCAGTCCTTGTGTG
        270       280       290       300       310       320

               1230      1240      1250      1260      1270      1280
human D5    CCAAGCGTGGCATAGAGTACCAGTCCAAGCCCCTGCTGTCAGCCTTCGCCGACATCATCC
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
2153526     CCAAGCATGGCATAGAGTACCAGTCCAAGCCCCTGCTGTCAGCCTTCGCCGACATCATCC
        330       340       350       360       370       380
```

FIG.28A

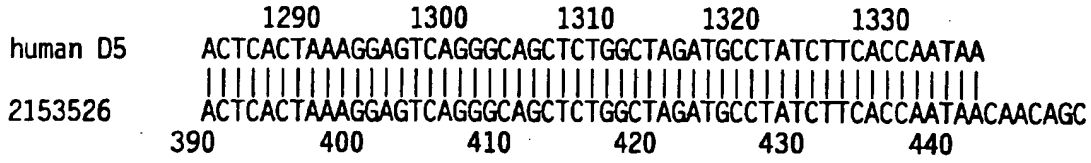
1290 1300 1310 1320 1330
human D5 ACTCACTAAAGGAGTCAGGGCAGCTCTGGCTAGATGCCTATCTTCACCAATAA
2153526 ACTCACTAAAGGAGTCAGGGCAGCTCTGGCTAGATGCCTATCTTCACCAATAACAACAGC
390 400 410 420 430 440

FIG.28B

FastA Match of Human D5-desaturase and
Contig 253538a

SCORES Init1: 1479 Initn: 2483 Opt: 2489
Smith-Waterman score: 2489; 81.3% identity in 434 aa overlap

```
                10        20        30        40        50        60
human D5    MAPDPVAAETAAQGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVIS
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
253538a             QGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVIS
                           10        20        30        40

                70        80        90       100       110       120
human D5    HYAGQDATDPFVAFHINKGLVKKYMNSLLIGELSPEQPSFEPTKNKELTDEFRELRATVE
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
253538a     HYAGQDATDPFVAFHINKGLVKKYMNSLLIGELSPEQPSFEPTKNKELTDEFRELRATVE
            50        60        70        80        90       100

               130       140       150       160       170       180
human D5    RMGLMKANHVFFLLYLLHILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQHD
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
253538a     RMGLMKANHVFFLLYLLHILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQHD
           110       120       130       140       150       160

               190       200       210       220       230       239
human D5    FGHLSVFSTSKWNHLLHHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPDINM-HPFFF
            :|||||:   |||||:|:|||||||| |:|||| |||||||| |:||||:|| |   |
253538a     YGHLSVYRKPKWNHLVHKFVIGHLKGASANWWNHRHFQHHAKPNIFHKDPDVNMLH--VF
           170       180       190       200       210       220

           240       250       260       270       280       290       299
human D5    ALGKILSVELGKQKKKYMPYNHQHKYFFLIGPPALLPLYFQWYIFYFVIQRKKWVDLAWM
            :||:   :| ||:| ||:|||||:|||||||| |:|:|||: |:: :| :|:||||||
253538a     VLGEWQPIEYGKKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIIMTMIVHKNWVDLAWA
             230       240       250       260       270       280

           300       310       320       330       340       350
human D5    ITFYVRFFLTYVPLLG-LKAFLGLFFIVRFLESNWFVWVTQMNHIPMHIDHDRNMDWVST
            :::|:|||:||:|: | | |:| | || |||||:|||||||||| |:||::   || |:
253538a     VSYYIRFFITYIPFYGILGALLFLNFI-RFLESHWFVWVTQMNHIVMEIDQEAYRDWFSS
             290       300       310       320       330       340

           360       370       380       390       400       410
human D5    QLLATCNVHKSAFNDWFSGHLNFQIEHHLFPTMPRHNYHKVAPLVQSLCAKRGIEYQSKP-
            || |||||::| |||||||||||||||||||||| ||:|||||:|||||:||||| ||
253538a     QLTATCNVEQSFFNDWFSGHLNFQIEHHLFPTMPRHNLHKIAPLVKSLCAKHGIEYQEKP
             350       360       370       380       390       400
```

FIG.29A

```
                420        430        440
human D5        LLSAFADIIHSLKESGQLWLDAYLHQX
                || |: |||:|||:||:|||||||:|
253538a         LLRALLDIIRSLKKSGKLWLDAYLHKXSHSPRDTVGKGCRWGDGQRNDGLLFXGVSERLV
                  410        420        430        440        450        460
```

FIG.29B

FastA Match of Human D5-desaturase and Contig 38

SCORES Init1: 2024 Initn: 2024 Opt: 2026
Smith-Waterman score: 2026; 99.3% identity in 287 aa overlap

```
                10        20        30        40        50        60
human D5    MAPDPVAAETAAQGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVIS
                          ||||||||||||||||||||||||||||||||||||||||||||||||||
38                     QGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVIS
                                  10        20        30        40

                70        80        90        100       110       120
human D5    HYAGQDATDPFVAFHINKGLVKKYMNSLLIGELSPEQPSFEPTKNKELTDEFRELRATVE
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38          HYAGQDATDPFVAFHINKGLVKKYMNSLLIGELSPEQPSFEPTKNKELTDEFRELRATVE
            50        60        70        80        90        100

                130       140       150       160       170       180
human D5    RMGLMKANHVFFLLYLLHILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQHD
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38          RMGLMKANHVFFLLYLLHILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQHD
            110       120       130       140       150       160

                190       200       210       220       230       240
human D5    FGHLSVFSTSKWNHLLHHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPDINMHPFFFA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
38          FGHLSVFSTSKWNHLLHHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPDINMHPFFFA
            170       180       190       200       210       220

                250       260       270       280       290       300
human D5    LGKILSVELGKQKKKYMPYNHQHKYFFLIGPPALLPLYFQWYIFYFVIQRKKWVDLAWMI
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||:
38          LGKILSVELGKQKKKYMPYNHQHXYFFLIGPPALLPLYFQWYIFYFVIQRKKWVDLAWIS
            230       240       250       260       270       280

                310       320       330       340       350       360
human D5    TFYVRFFLTYVPLLGLKAFLGLFFIVRFLESNWFVWVTQMNHIPMHIDHDRNMDWVSTQL

38          KQEYDEAGLPLSTANASKRDLPRATSPGTRWPSAQGARSGGXXSTVRCTTSASSPAGIQG
            290       300       310       320       330       340
```

FIG.30

FastA Match of D6-Desaturase (Ma524) and
Human D5-Desaturase

SCORES Init1: 280 Initn: 601 Opt: 303
Smith-Waterman score: 697; 30.5% identity in 455 aa overlap

```
                10        20        30        40        50
human D5    MAPDPVAAETAAQGPTPRYFTWDEV----AQRSGCEER----WLVIDRKVYNISEFTRRH
                 | :|: | ||  ||     |   | ::       ::|| |||:: ||:  |
Ma524.pep           MAAAPSVRTFTRAEVLNAEALNEGKKDAEAPFLMIIDNKVYDVREFVPDH
                         10        20        30        40        50

          60        70        80        90       100       110
human D5    PGGSRVISHYAGQDATDPFVAFHINKGLVKKYMNSLLIGELSPEQPSFEPTKNKELTDEF
            |||| :::| :|:|:|| | :|| : :   : : :: :|:::    | :  || ::: |
Ma524.pep   PGGSVILTH-VGKDGTDVFDTFHPEAAW--ETLANFYVGDIDE---SDRDIKNDDFAAEV
                   60        70        80        90       100

         120       130       140       150       160       170
human D5    RELRATVERMGLMKANHVFFLLYLLHILLLDGAAWLTLWVFG-TSFLPFLLCAVLLSAVQ
            |:||:  : :| : ::::::: : :   | : | : : :  :| || |  :| |:||:
Ma524.pep   RKLRTLFQSLGYYDSSKAYYAFKVSFNLCIWGLSTVIVAKWGQTSTLANVLSAALLGLFW
              110       120       130       140       150       160

          180       190       200       210       220       230
human D5    AQAGWLQHDFGHLSVFSTSKWNHLLHHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPD
             | ||| ||| | :||:   |: |:  |: | :|  :|||:  |  ||| ||   :|||
Ma524.pep   QQCGWLAHDFLHHQVFQDRFWGDLFGAFLGGVCQGFSSSWWKDKHNTHHAAPNVHGEDPD
              170       180       190       200       210       220

                240       250       260       270       280
human D5    INMHPFF----FALGKILSV---ELGKQKKKYMPYNHQHKYFFLIGPPALLPLYFQWYIF
            |: ||::     ||  : :|   || :: :::|  | |  ::| |   | |   :|  :|
Ma524.pep   IDTHPLLTWSEHALEMFSDVPDEELTRMWSRFMVLN-QTWFYFPILSFARLSWCLQSILF
              230       240       250       260       270       280

                        290       300       310       320       329
human D5    YFV------------IQRKKWVDLAWMITFYVRFFLTYV--PLLGLKAFLGLFFIVRFL
             :             |:  : ::||   |:|:  :: ::  |:     :|  |:: : :
Ma524.pep   VLPNGQAHKPSGARVPISLVEQLSLAMHWTWYLATMFLFIKDPV----NMLVYFLVSQAV
              290       300       310       320       330

          340       350       360       370       380
human D5    ESNWFVWVTQMNHIPMHI---DHDRNMDWVSTQLLATCNVHKSAFNDWFSGHLNFQIEHH
             :| :: | ::||  | :   ::  :||: : |:::  :|| : | :||:| ||:|||||
Ma524.pep   CGNLLAIVFSLNHNGMPVISKEEAVDMDFFTKQIITGRDVHPGLFANWFTGGLNYQIEHH
          340       350       360       370       380       390
```

FIG.31A

```
                390        400        410        420        430        440
human D5        LFPTMPRHNYHKVAPLVQSLCAKRGIEYQSKPLLSAFADIIHSLKESGQLWLDAYLHQX
                |||:|||||: |: | |::|| | :::|::  :: : |:::  |:|
Ma524.pep       LFPSMPRHNFSKIQPAVETLCKKYNVRYHTTGMIEGTAEVFSRLNEVSKAASKMGKAQX
              400        410        420        430        440        450
```

FIG.31B

FastA Match of D5-Desaturase (Ma29) and
Human D5-Desaturase

SCORES Init1: 145 Initn: 236 Opt: 266
Smith-Waterman score: 400; 27.5% identity in 455 aa overlap

```
                10        20        30        40        50        60
human D5    MAPDPVAAETAAQGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVIS
                 ||  |   |||:|:| ::  ::  |:|  :||::::|  |||||  ::
Ma29.pep          MGTDQGKT---FTWEELAAHNTKDDLLLAIRGRVYDVTKFLSRHPGGVDTLL
                           10        20        30        40

                70        80        90        100       110
human D5    HYAGQDATDPFVAFHINKGLVKKYMNSLLIGEL-SPEQPSF-EPTKNKELTDEFRELRAT
              ||:|:|  |  :|   | :   |::  :| | | | | | |||  ::      |   |
Ma29.pep    LGAGRDVTPVFEMYHAF-GAADAIMKKYYVGTLVSNELPIFPEPTVFHKTIKTRVEGYFT
           50        60        70        80        90        100

           120       130       140       150       160       170
human D5    VERMGLMKANHVF--FLLYLLHILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGW
             : :   :  :::  : | :  ::    |  :: :|   ::|  :: |:::: : ||:|
Ma29.pep    DRNIDPKNRPEIWGRYALIFGSLIASYYAQLFVPFVVERTWLQVVF-AIIMGFACAQVGL
           110       120       130       140       150       160

             180       190       200       210       220       230
human D5    LQ-HDFGHLSV-FSTSKWNHL--LHHFVIGHLKGAPASWWNHMH-FQHHAKPNCFRKDPD
              || :|:||  : : |: |   | |    ::||    | ::| : ||   |    |||
Ma29.pep    NPLHDASHFSVTHNPTVWKILGATHDF----FNGASYLVWMYQHMLGHHPYTNIAGADPD
            170       180       190           200       210       220

                240       250       260           270       280
human D5    INM-HPFFFALGKILSVELGKQKKKYMPYNH--QHKYF-FLIGPPALLPLYFQWYIFYFV
            ::  :|         :|:  | ::|::  ||  || :  || |  |:      :  |:|||
Ma29.pep    VSTSEP--------DVRRIKPNQKWF-VNHINQHMFVPFLYGLLAFKVRIQDINILYFV
                         230       240       250       260       270

            290       300       310           320       330
human D5    IQ----RKKWVDLAWMITFY--VRFFLTY---VPL--LGLKAFLGLFFIVRFLESNWFVW
                  | : ::    : |:    ||: |   |||  | |   | || :: :: | |::
Ma29.pep    KTNDAIRVNPISTWHTVMFWGGKAFFVWYRLIVPLQYLPLGKVLLLFTVADMVSSYWLAL
            280       290       300       310       320       330

             340       350       360       370       380
human D5    VTQMNHIPMHID---HDRN----MDWVSTQLLATCN-VHKSAFNDWFSGHLNFQIEHHLF
            : | ||:  :::    |:|     ||:: |: :| : :| | :   ::| ||:|  ||||
Ma29.pep    TFQANHVVEEVQWPLPDENGIIQKDWAAMQVETTQDYAHDSHLWTSITGSLNYQAVHHLF
            340       350       360       370       380       390
```

FIG.32A

```
                390        400        410        420        430        440
human D5        PTMPRHNYHKVAPLVQSLCAKRGIEYQSKPLL-SAFADIIHSLKESGQLWLDAYLHQX
                |:: :|:|  :  :::: |::  : |  |  : :|||: :: |:  |
Ma29.pep        PNVSQHHYPDILAIIKNTCSEYKVPYLVKDTFWQAFASHLEHLRVLGLRPKEEX
                    400        410        420        430        440
```

FIG.32B

HOST(PLASMID)	334(pRAE-28-5)	334(pRAE-26-1)	334(pRAE-33)	334(pRAE-35)	334(pYX242)
ADDED SUBSTRATE	25 μM DGLA	25 μM DGLA	25 μM DGLA	25 μM DGLA	25 μM DGLA
			30° C/48hrs		
FATTY ACID		(g FATTY ACID/100 g FATTY ACID)		lipid (μg)	
C16:0	151.580	202.175	285.291	281.298	304.229
C16:1	406.279	185.631	552.951	569.298	608.123
C18:0	16.494	25.995	32.162	27.479	30.093
C18:1n-9	100.031	133.349	173.772	184.740	187.780
C18:2n-6		0.180			0.946
C18:3n-6		0.058	0.074		0.074
C20:0	3.844	4.205	7.118	7.285	6.288
C20:3n-6	**96.576**	**118.657**	**134.859**	**139.292**	**125.448**
C20:4n-6	**(0.127%) 1.204**	**(0.075%) 0.878**	**(0.062%) 0.902**	**(0.063%) 0.927**	**(0.062%) 0.958**
C22:0		0.150	0.119		0.125
C22:1n-9	0.162	0.139	0.299	0.275	0.392
TOTAL LIPID	949.0	1169.0	1445.5	1468.0	1538.5

FIG.33

HOST(PLASMID)	334(pRAE-28-5)	334(pRAE-26-1)	334(pYX242)	334(pRAE-28-5)	334(pRAE-26-1)	334(pYX242)	334(pRAE-28-5)	334(pRAE-26-1)	334(pYX242)
ADDED SUBSTRATE	25 μM DGLA	25 μM DGLA	25 μM DGLA	25 μM OA	25 μM OA	25 μM OA	25 μM AA	25 μM AA	25 μM AA
			30 ℃/48hrs						30 ℃/48hrs
FATTY ACID	(g FATTY ACID/100 g FATTY ACID) lipid (μg)								
C16:0	49.332	106.358	93.225	84.327	37.013	51.018	78.471	53.685	74.099
C16:1	141.178	256.622	277.028	269.009	107.066	172.485	230.45	141.526	181.298
C18:0	9.301	14.819	12.908	11.871	8.3	9.047	11.283	9.97	10.969
C18:1n-9	39.876	87.564	72.842	106.416	52.634	71.453	61.754	42.289	46.873
C18:2n-6				ND	ND	ND			
C18:3n-6									
C20:0	2.154	7.339			3.729			2.685	
C20:3n-6	45.395	56.346	55.306						
C20:4n-6	(0.1062) 0.412	(0.0607) 0.396	(0.0653) 0.402				63.584	68.442	60.89
C22:5n-3							(0.0262) 0.139	(0.0192) 0.077	(0.0279) 0.126
TOTAL LIPID	387	665	620	562	284	363	535	404	466

FIG.34A

HOST(PLASMID)	334(pRAE-28-5)	334(pRAE-26-1)	334(pRAE-33)	334(pRAE-35)	334(pRAE-26-1)	334(pYX242)
ADDED SUBSTRATE	25 μM LA	25 μM LA	25 μM LA	NO SUBSTRATE	NO SUBSTRATE	NO SUBSTRATE
			30° C/48hrs			
FATTY ACID		(g FATTY ACID/100 g FATTY ACID)		lipid (μg)		
C16:0	56.631	45.393	74.247	174.138	25.574	33.44
C16:1	181.311	117.045	208.029	277.122	43.193	47.189
C18:0	9.549	9.251	11.45	22.547	5.119	8.432
C18:1n-9	48.256	46.496	51.342	134.822	21.89	32.618
C18:2n-6	31.91	23.221	36.821			
C18:3n-6	(0.02%) 0.082	ND	(0.012%) 0.056			
C20:0		0.339		0.702		
C20:3n-6						
C20:4n-6						
C20:5n-3	0.121					
TOTAL LIPID	407	279	460	746	127	168

FIG.34B

HUMAN DESATURASE GENE AND USES THEREOF

The subject application is a Continuation-In-Part of pending U.S. patent application Ser. No. 09/227,613 filed on Jan. 8, 1999, which is a Continuation-In-Part of pending International Application PCT/US98/07422 filed on Apr. 10, 1998 (which designates the U.S.) which is a Continuation-In-Part of U.S. patent application Ser. No. 08/833,610 filed on Apr. 11, 1997, now U.S. Pat. No. 5,972,664 all of which are incorporated herein in their entirety by reference.

BACKGROUND OF THE INVENTION

1. Technical Field

The subject invention relates to the identification and isolation of a gene that encodes an enzyme (i.e., human Δ5-desaturase) involved in the synthesis of polyunsaturated fatty acids and to uses thereof. In particular, Δ5-desaturase VQ catalyzes the conversion of, for example, dihomo-γ-linolenic acid (DGLA) to arachidonic acid (AA) and (n-3)-eicosatetraenoic acid (20:4n-3) to eicosapentaenoic acid (20:5n-3). The converted product may then be utilized as a substrate in the production of other polyunsaturated fatty acids (PUFAs). The product or other polyunsaturated fatty acids may be added to pharmaceutical compositions, nutritional composition, animal feeds as well as other products such as cosmetics.

2. Background Information

Desaturases are critical in the production of long-chain polyunsaturated fatty acids which have many important functions. For example, PUFAs are important components of the plasma membrane of a cell, where they are found in the form of phospholipids. They also serve as precursors to mammalian prostacyclins, eicosanoids, leukotrienes and prostaglandins. Additionally, PUFAs are necessary for the proper development of the developing infant brain as well as for tissue formation and repair. In view of the biological significance of PUFAs, attempts are being made to produce them, as well as intermediates leading to their production, in an efficient manner.

A number of enzymes are involved in PUFA biosynthesis including Δ5-desaturase (see FIG. 11). For example, elongase (elo) catalyzes the conversion of γ-linolenic acid (GLA) to dihomo-γ-linolenic acid (DGLA) and of stearidonic acid (18:4n-3) to (n-3)-eicosatetraenoic acid (20:4n-3). Linoleic acid (LA, 18:2-Δ9,12 or 18:2n-6) is produced from oleic acid (18:1-Δ9) by a Δ12-desaturase. GLA (18:3-Δ6,9,12) is produced from linoleic Δ5 acid by a Δ6-desaturase.

It must be noted that animals cannot desaturate beyond the Δ9 position and therefore cannot convert oleic acid into linoleic acid. Likewise, α-linolenic acid (ALA, 18:3-Δ9,12, 15) cannot be synthesized by mammals. However, α-linolenic acid can be converted to stearidonic acid (STA, 18:4-Δ6,9,12,15) by a Δ6-desaturase (see PCT publication WO 96/13591 and *The Faseb Journal,* Abstracts, Part I, Abstract 3093, page A532 (Experimental Biology 98, San Francisco, Calif., Apr. 18–22, 1998) see also U.S. Pat. No. 5,552,306), followed by elongation to (n-3)-eicosatetraenoic acid (20:4-Δ8,11,14,17) in mammals and algae. This polyunsaturated fatty acid (i.e., 20:4-Δ8,11,14,17) can then be converted to eicosapentaenoic acid (EPA, 20:5-Δ5,8,11,14, 17) by a Δ5-desaturase, such as that of the present invention. Other eukaryotes, including fungi and plants, have enzymes which desaturate at carbon 12 (see PCT publication WO 94/11516 and U.S. Pat. No. 5,443,974) and carbon 15 (see PCT publication WO 93/11245). The major polyunsaturated

fatty acid of animals therefore are either derived from diet and/or from desaturation and elongation of linoleic acid or α-linolenic acid. In view of these difficulties, it is of significant interest to isolate genes involved in PUFA synthesis from species that naturally produce these fatty acids and to express these genes in a microbial, plant, or animal system which can be altered to provide production of commercial quantities of one or more PUFAs. One of the most important long chain PUFAs, noted above, is arachidonic acid (AA). AA is found in filamentous fungi and can also be purified from mammalian tissues including the liver and adrenal glands. As noted above, AA production from dihomo-γ-linolenic acid is catalyzed by a Δ5-desaturase. EPA is another important long-chain PUFA. EPA is found in fungi and also in marine oils. As noted above, EPA is produced from (n-3)-eicosatetraenoic acid and is catalyzed by a Δ5-desaturase.

In view of the above discussion, there is a definite need for the Δ5-desaturase enzyme, the gene encoding this enzyme, as well as recombinant methods of producing this enzyme. Additionally, a need exists for oils containing levels of PUFAs beyond those naturally present as well as those enriched in novel PUFAs. Such oils can only be made by isolation and expression of the Δ5-desaturase gene.

All U.S. patents and publications referred to herein are hereby incorporated in their entirety by reference.

SUMMARY OF THE INVENTION

The present invention includes an isolated nucleotide sequence corresponding to or complementary to at least about 50% of the nucleotide sequence shown in SEQ ID NO:1 (FIG. 12). The isolated nucleotide sequence may be represented by SEQ ID NO:1. These sequences may encode a functionally active desaturase which utilizes a polyunsaturated fatty acid as a substrate. The sequences may be derived from a mammal such as, for example, a human.

The present invention also includes purified proteins encoded by the nucleotide sequences referred to above. Additionally, the present invention includes a purified polypeptide which desaturates polyunsaturated fatty acids at carbon 5 and has at least about 50% amino acid similarity to the amino acid sequence of the purified proteins referred to directly above.

Furthermore, the present invention also encompasses a method of producing a human Δ5-desaturase. This method comprises the steps of: a) isolating the nucleotide sequence represented by SEQ ID NO:1 (FIG. 12); b) constructing a vector comprising: i) the isolated nucleotide sequence operably linked to ii) a promoter; and c) introducing the vector into a host cell under time and conditions sufficient for expression of the human Δ5-desaturase. The host cell may be, for example, a eukaryotic cell or a prokaryotic cell. In particular, the prokaryotic cell may be, for example, *E. coli,* cyanobacteria or *B. subtilis.* The eukaryotic cell may be, for example, a mammalian cell, an insect cell, a plant cell or a fungal cell (e.g., a yeast cell such as *Saccharomyces cerevisiae, Saccharomyces carlsbergensis,* Candida spp., *Lipomyces starkey, Yarrowia lipolytica,* Kluvveromyces spp., Hansemula Spp., Trichoderma spp. or Pichia spp.).

Additionally, the present invention also encompasses a vector comprising: a) a nucleotide sequence as represented by SEQ ID NO:1 (FIG. 12) operably linked to b) a promoter. The invention also includes a host cell comprising this vector. The host cell may be, for example, a eukaryotic cell or a prokaryotic cell. Suitable eukaryotic cells and prokaryotic cells are as defined above.

Moreover, the present invention also includes a plant cell, plant or plant tissue comprising the above vector, wherein expression of the nucleotide sequence of the vector results in production of a polyunsaturated fatty acid by the plant cell, plant or plant tissue. The polyunsaturated fatty acid may be, for example, selected from the group consisting of AA and EPA. The invention also includes one or more plant oils or acids expressed by the above plant cell, plant or plant tissue.

Additionally, the present invention also encompasses a transgenic plant comprising the above vector, wherein expression of the nucleotide sequence of the vector results in production of a polyunsaturated fatty acid in seeds of the transgenic plant.

Also, the invention includes a mammalian cell comprising the above vector wherein expression of the nucleotide sequence of the vector results in production of altered levels of AA or EPA when the cell is grown in a culture media comprising a fatty acid selected from the group consisting of an essential fatty acid, LA and ALA.

It should also be noted that the present invention encompasses a transgenic, non-human mammal whose genome comprises a DNA sequence encoding a human Δ5-desaturase operably linked to a promoter. The DNA sequence may be represented by SEQ ID NO:1 (FIG. 12). Additionally, the present invention includes a fluid (e.g., milk) produced by the transgenic, non-human mammal wherein the fluid comprises a detectable level of at least human Δ5-desaturase.

Additionally, the present invention includes a method (i.e., "first" method) for producing a polyunsaturated fatty acid comprising the steps of: a) isolating the nucleotide sequence represented by SEQ ID NO:1 (FIG. 12); b) constructing a vector comprising the isolated nucleotide sequence; c) introducing the vector into a host cell under time and conditions sufficient for expression of the human Δ5-desaturase enzyme; and d) exposing the expressed human Δ5-desaturase enzyme to a substrate polyunsaturated fatty acid in order to convert the substrate to a product polyunsaturated fatty acid. The substrate polyunsaturated fatty acid may be, for example, DGLA or 20:4n-3 and the product polyunsaturated fatty acid may be, for example, AA or EPA, respectively. This method may further comprise the step of exposing the product polyunsaturated fatty acid to an elongase in order to convert the product polyunsaturated fatty acid to another polyunsaturated fatty acid (i.e., "second" method). In this method containing the additional step (i.e., "second" method), the product polyunsaturated fatty acid may be, for example, AA or EPA, and the "another" polyunsaturated fatty acid may be adrenic acid or (n-3)-docosapentaenoic acid, respectively. The method containing the additional step may further comprise a step of exposing the another polyunsaturated fatty acid to an additional desaturase in order to convert the another polyunsaturated fatty acid to a final polyunsaturated fatty acid (i.e., "third" method). The final polyunsaturated fatty acid may be, for example, (n-6)-docosapentaenoic acid or docosahexaenoic (DHA) acid.

The present invention also encompasses a nutritional composition comprising at least one polyunsaturated fatty acid selected from the group consisting of the product polyunsaturated fatty acid produced according to the "first" method, another polyunsaturated fatty acid produced according to the "second" method, and the final polyunsaturated fatty acid produced according to the "third" method. The product polyunsaturated fatty acid may be, for example, AA or EPA. The another polyunsaturated fatty acid may be, for example, adrenic acid or (n-3)-docosapentaenoic acid. The final polyunsaturated fatty acid may be, for example, (n-6)-docosapentaenoic acid or DHA. This nutritional composition, may be, for example, an infant formula, a dietary supplement or a dietary substitute and may be administered to a human or to an animal. It may be administered enterally or parenterally. The nutritional composition may further comprise at least one macronutrient selected from the group consisting of coconut oil, soy oil, canola oil, monoglycerides, diglycerides, glucose, edible lactose, electrodialysed whey, electrodialysed skim milk, milk whey, soy protein, and protein hydrolysates. Additionally, the composition may further comprise at least one vitamin selected from the group consisting of Vitamins A, C, D, E, and B complex and at least one mineral selected from the group consisting of calcium magnesium, zinc, manganese, sodium, potassium, phosphorus, copper, chloride, iodine, selenium and iron.

Furthermore, the present invention also includes a a pharmaceutical composition comprising 1) at least one polyunsaturated fatty acid selected from the group consisting of the product polyunsaturated fatty acid produced according to the "first" method, the another polyunsaturated fatty acid produced according to the "second" method, and the final polyunsaturated fatty acid produced according to the "third" method and 2) a pharmaceutically acceptable carrier. Again, the pharmaceutical composition may be administered to a human or to an animal. The composition may further comprise an element selected from the group consisting of a vitamin, a mineral, a carbohydrate, an amino acid, a free fatty acid, a phospholipid, an antioxidant, and a phenolic compound.

Additionally, the present invention includes an animal feed comprising at least one polyunsaturated fatty acid selected from the group consisting of the product polyunsaturated fatty acid produced according to the first method, the another polyunsaturated fatty acid produced according to the second method and the final polyunsaturated fatty acid produced according to the third method. The product polyunsaturated fatty acid may be, for example, AA or EPA. The another polyunsaturated fatty acid may be, for example, adrenic acid or (n-3)-docosapentaenoic acid. The final polyunsaturated fatty acid may be, for example, (n-6)-docosapentaenoic acid or DHA.

Moreover, the present invention also includes a cosmetic comprising a polyunsaturated fatty acid selected from the group consisting of the product polyunsaturated fatty acid produced according to the first method, the another polyunsaturated fatty acid produced according to the second method, and the final polyunsaturated fatty acid produced according to the third method.

Additionally, the present invention encompasses a method of preventing or treating a condition caused by insufficient intake of polyunsaturated fatty acids comprising administering to the patient the nutritional composition of above in an amount sufficient to effect prevention or treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 outlines the sections of the *M. alpina* Δ5- and Δ6-desaturases, the clone ID's from the LifeSeq database to which those sections had homology, and the keyword associated with the clone ID's.

FIG. 2 represents the contig 2692004 (SEQ ID NO:2).

FIG. 3 represents the contig 2153526 (SEQ ID NO:3).

FIG. 4 represents the contig 3506132 (SEQ ID NO:4).

FIG. 5 represents the contig 3854933 (SEQ ID NO:5).

FIG. 6 represents the contig 2511785 (SEQ ID NO:6).

FIG. 7 represents the contig 2535 (SEQ ID NO:7) generated based on contig 2511785 of FIG. 6 and contig 3506132 of FIG. 4.

FIG. 8 represents the contig 253538a (SEQ ID NO:8) generated based on contig 2535 of FIG. 7 and contig 3854933 of FIG. 5.

FIG. 9 represents the amino acid sequence identity between the *M. alpina* Δ5-desaturase (Ma29) and the contig 253538a (SEQ ID NO:9).

FIG. 10 represents the amino acid sequence identity between the *M. alpina* Δ6-desaturase (Ma524) (SEQ ID NO:10) and the contig 253538a (SEQ ID NO:9).

FIG. 11 represents various fatty acid biosynthesis pathways. The role of the Δ5-desaturase enzyme should be noted.

FIG. 12 represents the complete nucleotide sequence of the human Δ5-desaturase gene (human Δ5) (SEQ ID NO:1).

FIG. 13 represents the amino acid sequence of the human Δ5-desaturase (SEQ ID NO:11) translated from human Δ5 (see FIG. 12).

FIG. 14 illustrates the sequence identity between the pRAE-7 and pRAE-8 clones.

FIG. 15 represents the complete putative human desaturase gene sequence (SEQ ID NO:12) from clone pRAE-7 and the corresponding, translated amino acid sequence (SEQ ID NO:13).

FIG. 16 illustrates the amino acid sequence identity between the putative human desaturase gene in pRAE-7 (SEQ ID NO:14) and the *M. alpina* Δ5-desaturase (SEQ ID NO:15).

FIG. 17 illustrates the amino acid sequence identity between the putative human desaturase gene in pRAE-7 (SEQ ID NO:16) and the *M. alpina* Δ6-desaturase (SEQ ID NO:18).

FIG. 18 illustrates the amino acid sequence identity between the putative human desaturase gene in pRAE-7 (SEQ ID NO:18) and the contig 2535 (SEQ ID NO:19).

FIG. 19 illustrates the amino acid sequence identity between the putative human desaturase gene in pRAE-7 (SEQ ID NO:20) and the contig 38 (SEQ ID NO:21).

FIG. 20 illustrates the amino acid sequence identity between the N-terminus of clone A-1 (SEQ ID NO:22), a representative of Group 1, and the N-terminus of the cytochrome b5 gene.

FIG. 21 illustrates the nucleotide sequence identity between the nucleotide sequence of a portion of clone A-1 (SEQ ID NO:24) and a portion of the GenBank sequence ac004228 (SEQ ID NO:25).

FIG. 22 represents the nucleotide sequence identity between the nucleotide sequence of a portion of clone 3-5 (SEQ ID NO:26) of Group 2 and a portion of the GenBank sequence acO04228 (SEQ ID NO:27). Clone 3-5 has an ATG within a NcoI site, but translates four stops between the ATG and the BamHI site.

FIG. 23 represents the nucleotide sequence identity between the nucleotide sequence of a portion of clone A-10 (SEQ ID NO:28) of Group 3 and a portion of the GenBank sequence ac004228 (SEQ ID NO:29). Clone A-10 has an ATG 135 bp upstream of the BamHI site, giving an open reading frame of 1267 bp.

FIG. 24 represents the nucleotide sequence identity between the nucleotide sequence of a portion of clone A-16 (SEQ ID NO:30) of Group 4 and a portion of the GenBank sequence ac004228 (SEQ ID NO:31). Clone A-16 does not have an ATG; however, there is an ATG (underlined) upstream of where the sequence aligns with ac004228.

FIG. 25 represents the nucleotide sequence identity between the nucleotide sequence of a portion of clone A-19 (SEQ ID NO:32) of Group 5 and a portion of the GenBank sequence ac004228 (SEQ ID NO:33). Clone A-19 does not have an ATG; however, this clone matches the ac004228 sequence even upstream of the BamHI site.

FIG. 26 represents the partial nucleotide sequence of the GenBank sequence ac004228 and the representative clones from the five Groups (SEQ ID NO:34).

FIG. 27 represents the nucleotide sequence identity between the human Δ5-desaturase (SEQ ID NO:35) and contig 3381584 (SEQ ID NO:36).

FIG. 28 represents the nucleotide sequence identity between the human Δ5-desaturase (SEQ ID NO:37) and contig 2153526 (SEQ ID NO:38).

FIG. 29 represents the amino acid sequence identity between the human Δ5-desaturase (SEQ ID NO:39) and contig 253538a (SEQ ID NO:40).

FIG. 30 represents the amino acid sequence identity between the human Δ5-desaturase (SEQ ID NO:41) and contig 38 (SEQ ID NO:42).

FIG. 31 represents the amino acid sequence identity between the *M. alpina* Δ6-desaturase (Ma524) (SEQ ID NO:44) and the human the Δ5-desaturase (SEQ ID NO:43).

FIG. 32 represents the amino acid sequence identity between the *M. alpina* Δ5-desaturase (Ma29) (SEQ ID NO:46) and the human Δ5-desaturase (SEQ ID NO:45).

FIG. 33 illustrates the human Δ5-desaturase activity of the gene in clone pRAE-28-5, compared to that in pRAE-26-1, pRAE-33, and pRAE-35, when expressed in baker's yeast.

FIG. 34 illustrates the substrate specificity of the human Δ5-desaturase gene in clone pRAE-28-5, converting DGLA (20:3n-6) to AA (20:4n-6), when the gene is expressed in baker's yeast.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention relates to the nucleotide and amino acid sequence of the Δ5-desaturase gene derived from humans. Furthermore, the subject invention also includes uses of the gene and of the enzyme encoded by this gene. For example, the gene and corresponding enzyme may be used in the production of polyunsaturated fatty acids such as, for instance, arachidonic acid, eicosapentaenoic acid, and/or adrenic acid which may be added to pharmaceutical compositions, nutritional compositions and to other valuable products.

The Human Δ5-Desaturase Gene and Enzyme Encoded Thereby

As noted above, the enzyme encoded by the human Δ5-desaturase gene is essential in the production of highly unsaturated polyunsaturated fatty acids having a length greater than 20 carbons. The nucleotide sequence of the isolated human Δ5-desaturase gene is shown in FIG. 2, and the amino acid sequence of the corresponding purified protein is shown in FIG. 3.

As an example, the isolated human Δ5-desaturase gene of the present invention converts DGLA to AA or converts 20:4n-3 to EPA. Thus, neither AA nor EPA, for example, can be synthesized without the Δ5-desaturase gene (e.g., human or *M. alpina*) and enzyme encoded thereby.

It should be noted that the present invention also encompasses nucleotide sequences (and the corresponding

encoded proteins) having sequences corresponding to or complementary to at least about 50%, preferably at least about 60%, and more preferably at least about 70% of the nucleotides in sequence to SEQ ID NO:1 (i.e., the nucleotide sequence of the human Δ5-desaturase gene described herein (see FIG. 12)). Such sequences may be derived from non-human sources (e.g., *C. elegans* or mouse). Furthermore, the present invention also encompasses fragments and derivatives of the nucleotide sequence of the present invention (i.e., SEQ ID NO:1), as well as of the sequences derived from non-human sources, and having the above-described complementarity or correspondence. Functional equivalents of the above-sequences (i.e., sequences having human Δ5-desaturase activity) are also encompassed by the present invention. The invention also includes a purified polypeptide which desaturates polyunsaturated fatty acids at the carbon 5 position and has at least about 50% amino acid similarity to the amino acid sequence of the above-noted proteins which are, in turn, encoded by the above-described nucleotide sequences.

The present invention also encompasses an isolated nucleotide sequence which encodes PUFA desaturase activity and that is hybridizable, under moderately stringent conditions, to a nucleic acid having a nucleotide sequence corresponding to or complementary to the nucleotide sequence represented by SEQ ID NO:1 and shown in FIG. 12. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule when a single-stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and ionic strength (see Sambrook et al., "Molecular Cloning: A Laboratory Manual, Second Edition (1989), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.)). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. "Hybridization" requires that two nucleic acids contain complementary sequences. However, depending on the stringency of the hybridization, mismatches between bases may occur. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation. Such variables are well known in the art. More specifically, the greater the degree of similarity or homology between two nucleotide sequences, the greater the value of Tm for hybrids of nucleic acids having those sequences. For hybrids of greater than 100 nucleotides in length, equations for calculating Tm have been derived (see Sambrook et al., supra). For hybridization with shorter nucleic acids, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., supra).

Production of the Human Δ5-Desaturase Enzyme

Once the gene encoding the human Δ5-desaturase enzyme has been isolated, it may then be introduced into either a prokaryotic or eukaryotic host cell through the use of a vector or construct.

The vector, for example, a bacteriophage, cosmid or plasmid, may comprise the nucleotide sequence encoding the human Δ5-desaturase enzyme as well as any promoter which is functional in the host cell and is able to elicit expression of the human Δ5-desaturase encoded by the nucleotide sequence. The promoter is in operable association with or operably linked to the nucleotide sequence. (A promoter is said to be "operably linked" with a coding sequence if the promoter affects transcription or expression of the coding sequence.) Suitable promoters include, for example, those from genes encoding alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglucoisomerase, phosphoglycerate kinase, acid phosphatase, T7, TPI, lactase, metallothionein, cytomegalovirus immediate early, whey acidic protein, glucoamylase, and promoters activated in the presence of galactose, for example, GAL1 and GAL10. Additionally, nucleotide sequences which encode other proteins, oligosaccharides, lipids, etc. may also be included within the vector as well as other regulatory sequences such as a polyadenylation signal (e.g., the poly-A signal of SV-40T-antigen, ovalalbumin or bovine growth hormone). The choice of sequences present in the construct is dependent upon the desired expression products as well as the nature of the host cell.

As noted above, once the vector has been constructed, it may then be introduced into the host cell of choice by methods known to those of ordinary skill in the art including, for example, transfection, transformation and electroporation (see *Molecular Cloning: A Laboratory Manual,* 2nd ed., Vol. 1–3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press (1989)). The host cell is then cultured under suitable conditions permitting expression of the desired PUFA which is then recovered and purified.

Examples of suitable prokaryotic host cells include, for example, bacteria such as *Escherichia coli, Bacillus subtilis* as well as cyanobacteria such as Spirulina spp. (i.e., blue-green algae). Examples of suitable eukaryotic host cells include, for example, mammalian cells, plant cells, yeast cells such as *Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Lipomyces starkey,* Candida spp. such as *Yarrowia* (Candida) *lipolytica,* Kluyveromyces spp., Pichia spp., Trichoderma Spp. or Hansenula spp., or fungal cells such as filamentous fungal cells, for example, Aspergillus, Neurospora and Penicillium. Preferably, *Saccharomyces cerevisiae* (baker's yeast) cells are utilized.

Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of expression. Stable expression can be achieved by introduction of a construct that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression construct, followed by selection for cells expressing the marker. When stable expression results from integration, the site of the construct's integration can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

A transgenic mammal may also be used in order to express the enzyme of interest (i.e., the human Δ5-desaturase), and ultimately the PUFA(s) of interest. More specifically, once the above-described construct is created, it may be inserted into the pronucleus of an embryo. The embryo may then be implanted into a recipient female. Alternatively, a nuclear transfer method could also be utilized (Schnieke et al., *Science* 278:2130–2133 (1997)). Gestation and birth are then permitted (see, e.g., U.S. Pat. No. 5,750,176 and U.S. Pat. No. 5;700,671). Milk, tissue or

other fluid samples from the offspring should then contain altered levels of PUFAs, as compared to the levels normally found in the non-transgenic animal. Subsequent generations may be monitored for production of the altered or enhanced levels of PUFAs and thus incorporation of the gene encoding the human Δ5-desaturase enzyme into their genomes. The mammal utilized as the host may be selected from the group consisting of, for example, a mouse, a rat, a rabbit, a pig, a goat, a sheep, a horse and a cow. However, any mammal may be used provided it has the ability to incorporate DNA encoding the enzyme of interest into its genome.

For expression of a human Δ5-desaturase polypeptide, functional transcriptional and translational initiation and termination regions are operably linked to the DNA encoding the desaturase polypeptide. Transcriptional and translational initiation and termination regions are derived from a variety of nonexclusive sources, including the DNA to be expressed, genes known or suspected to be capable of expression in the desired system, expression vectors, chemical synthesis, or from an endogenous locus in a host cell. Expression in a plant tissue and/or plant part presents certain efficiencies, particularly where the tissue or part is one which is harvested early, such as seed, leaves, fruits, flowers, roots, etc. Expression can be targeted to that location with the plant by utilizing specific regulatory sequence such as those of U.S. Pat. Nos. 5,463,174, 4,943,674, 5,106,739, 5,175,095, 5,420,034, 5,188,958, and 5,589,379. Alternatively, the expressed protein can be an enzyme which produces a product which may be incorporated, either directly or upon further modifications, into a fluid fraction from the host plant. Expression of a human Δ5-desaturase gene, or antisense human Δ5-desaturase transcripts, can alter the levels of specific PUFAs, or derivatives thereof, found in plant parts and/or plant tissues. The human Δ5-desaturase polypeptide coding region may be expressed either by itself or with other genes, in order to produce tissues and/or plant parts containing higher proportions of desired PUFAs or in which the PUFA composition more closely resembles that of human breast milk (Prieto et al., PCT publication WO 95/24494). The termination region may be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known to and have been found to be satisfactory in a variety of hosts from the same and different genera and species. The termination region usually is selected as a matter of convenience rather than because of any particular property.

As noted above, a plant (e.g., *Glycine max* (soybean) or *Brassica napus* (canola)) or plant tissue may also be utilized as a host or host cell, respectively, for expression of the human Δ5-desaturase enzyme which may, in turn, be utilized in the production of polyunsaturated fatty acids. More specifically, desired PUFAS can be expressed in seed. Methods of isolating seed oils are known in the art. Thus, in addition to providing a source for PUFAs, seed oil components may be manipulated through the expression of the human Δ5-desaturase gene, as well as perhaps other desaturase genes and elongase genes, in order to provide seed oils that can be added to nutritional compositions, pharmaceutical compositions, animal feeds and cosmetics. Once again, a vector which comprises a DNA sequence encoding the human Δ5-desaturase operably linked to a promoter, will be introduced into the plant tissue or plant for a time and under conditions sufficient for expression of the human Δ5-desaturase gene. The vector may also comprise one or more genes that encode other enzymes, for example, Δ4-desaturase, elongase, Δ6-desaturase, Δ12-desaturase, Δ15-desaturase, Δ17-desaturase, and/or Δ19-desaturase. The plant tissue or plant may produce the relevant substrate (e.g., DGLA, GLA, EPA, 20:4n-3, etc.) upon which the enzymes act or a vector encoding enzymes which produce such substrates may be introduced into the plant tissue, plant cell or plant. In addition, substrate may be sprayed on plant tissues expressing the appropriate enzymes. Using these various techniques, one may produce PUFAs (e.g., n-6 unsaturated fatty acids such as AA, or n-3 fatty acids such as EPA or DHA) by use of a plant cell, plant tissue or plant. It should also be noted that the invention also encompasses a transgenic plant comprising the above-described vector, wherein expression of the nucleotide sequence of the vector results in production of a polyunsaturated fatty acid in, for example, the seeds of the transgenic plant.

The substrates which may be produced by the host cell either naturally or transgenically, as well as the enzymes which may be encoded by DNA sequences present in the vector which is subsequently introduced into the host cell, are shown in FIG. 11.

In view of the above, the present invention encompasses a method of producing the human Δ5-desaturase enzyme comprising the steps of: 1) isolating the nucleotide sequence of the gene encoding human Δ5-desaturase enzyme; 2) constructing a vector comprising said nucleotide sequence; and 3) introducing said vector into a host cell under time and conditions sufficient for the production of the desaturase enzyme.

The present invention also encompasses a method of producing polyunsaturated fatty acids comprising exposing an acid to the human Δ5-desaturase enzyme such that the desaturase converts the acid to a polyunsaturated fatty acid. For example, when 20:3n-6 is exposed to human Δ5-desaturase enzyme, it is converted to AA. AA may then be exposed to elongase which elongates the AA to adrenic acid (i.e., 22:4n-6). Alternatively, human Δ5-desaturase may be utilized to convert 20:4n-3 to 20:5n-3 which may be exposed to elongase and converted to (n-3)-docosapentaenoic acid. The (n-3)-docosapentaenoic acid may then be converted to DHA by use of Δ4-desaturase. Thus, human Δ5-desaturase may be used in the production of polyunsaturated fatty acids which may be used, in turn, for particular beneficial purposes.

Uses of the Human Δ5-Desaturase Gene and Enzyme Encoded Thereby

As noted above, the isolated human Δ5-desaturase gene and the desaturase enzyme encoded thereby have many uses. For example, the gene and corresponding enzyme may be used indirectly or directly in the production of polyunsaturated fatty acids, for example, AA, adrenic acid or EPA. ("Directly" is meant to encompass the situation where the enzyme directly converts the acid to another acid, the latter of which is utilized in a composition (e.g., the conversion of DGLA to AA). "Indirectly" is meant to encompass the situation where an acid is converted to another acid (i.e., a pathway intermediate) by the desaturase (e.g., DGLA to AA) and then the latter acid is converted to another acid by use of a non-desaturase enzyme (e.g., AA to adrenic acid by elongase or by use of another desaturase enzyme (e.g., AA to EPA by Δ17-desaturase.)). These polyunsaturated fatty acids (i.e., those produced either directly or indirectly by activity of the desaturase enzyme) may be added to, for example, nutritional compositions, pharmaceutical compositions, cosmetics, and animal feeds, all of which are encompassed by the present invention. These uses are described, in detail, below.

Nutritional Compositions

The present invention includes nutritional compositions. Such compositions, for purposes of the present invention, include any food or preparation for human consumption including for enteral or parenteral consumption, which when taken into the body (a) serve to nourish or build up tissues or supply energy and/or (b) maintain, restore or support adequate nutritional status or metabolic function.

The nutritional composition of the present invention comprises at least one oil or acid produced directly or indirectly by use of the human Δ5-desaturase gene, in accordance with the present invention, and may either be in a solid or liquid form. Additionally, the composition may include edible macronutrients, vitamins and minerals in amounts desired for a particular use. The amount of such ingredients will vary depending on whether the composition is intended for use with normal, healthy infants, children or adults having specialized needs such as those which accompany certain metabolic conditions (e.g., metabolic disorders).

Examples of macronutrients which may be added to the composition include but are not limited to edible fats, carbohydrates and proteins. Examples of such edible fats include but are not limited to coconut oil, soy oil, and mono- and diglycerides. Examples of such carbohydrates include but are not limited to glucose, edible lactose and hydrolyzed search. Additionally, examples of proteins which may be utilized in the nutritional composition of the invention include but are not limited to soy proteins, electrodialysed whey, electrodialysed skim milk, milk whey, or the hydrolysates of these proteins.

With respect to vitamins and minerals, the following may be added to the nutritional compositions of the present invention: calcium, phosphorus, potassium, sodium, chloride, magnesium, manganese, iron, copper, zinc, selenium, iodine, and Vitamins A, E, D, C, and the B complex. Other such vitamins and minerals may also be added.

The components utilized in the nutritional compositions of the present invention will be of semi-purified or purified origin. By semi-purified or purified is meant a material which has been prepared by purification of a natural material or by synthesis.

Examples of nutritional compositions of the present invention include but are not limited to infant formulas, dietary supplements, dietary substitutes, and rehydration compositions. Nutritional compositions of particular interest include but are not limited to those utilized for enteral and parenteral supplementation for infants, specialist infant formulas, supplements for the elderly, and supplements for those with gastrointestinal difficulties and/or malabsorption.

The nutritional composition of the present invention may also be added to food even when supplementation of the diet is not required. For example, the composition may be added to food of any type including but not limited to margarines, modified butters, cheeses, milk, yogurt, chocolate, candy, snacks, salad oils, cooking oils, cooking fats, meats, fish and beverages.

In a preferred embodiment of the present invention, the nutritional composition is an enteral nutritional product, more preferably, an adult or pediatric enteral nutritional product. This composition may be administered to adults or children experiencing stress or having specialized needs due to chronic or acute disease states. The composition may comprise, in addition to polyunsaturated fatty acids produced in accordance with the present invention, macronutrients, vitamins and minerals as described above. The macronutrients may be present in amounts equivalent to those present in human milk or on an energy basis, i.e., on a per calorie basis.

Methods for formulating liquid or solid enteral and parenteral nutritional formulas are well known in the art. (See also the Examples below.)

The enteral formula, for example, may be sterilized and subsequently utilized on a ready-to-feed (RTF) basis or stored in a concentrated liquid or powder. The powder can be prepared by spray drying the formula prepared as indicated above, and reconstituting it by rehydrating the concentrate. Adult and pediatric nutritional formulas are well known in the art and are commercially available (e.g., Similac®, Ensure®, Jevity® and Alimentum® from Ross Products Division, Abbott Laboratories, Columbus, Ohio). An oil or acid produced in accordance with the present invention may be added to any of these formulas.

The energy density of the nutritional compositions of the present invention, when in liquid form, may range from about 0.6 Kcal to about 3 Kcal per ml. When in solid or powdered form, the nutritional supplements may contain from about 1.2 to more than 9 Kcals per gram, preferably about 3 to 7 Kcals per gm. In general, the osmolality of a liquid product should be less than 700 mOsm and, more preferably, less than 660 mOsm.

The nutritional formula may include macronutrients, vitamins, and minerals, as noted above, in addition to the PUFAs produced in accordance with the present invention. The presence of these additional components helps the individual ingest the minimum daily requirements of these elements. In addition to the provision of PUFAs, it may also be desirable to add zinc, copper, folic acid and antioxidants to the composition. It is believed that these substance boost a stressed immune system and will therefore provide further benefits to the individual receiving the composition. A pharmaceutical composition may also be supplemented with these elements.

In a more preferred embodiment, the nutritional composition comprises, in addition to antioxidants and at least one PUFA, a source of carbohydrate wherein at least 5 weight percent of the carbohydrate is indigestible oligosaccharide. In a more preferred embodiment, the nutritional composition additionally comprises protein, taurine, and carnitine.

As noted above, the PUFAs produced in accordance with the present invention, or derivatives thereof, may be added to a dietary substitute or supplement, particularly an infant formula, for patients undergoing intravenous feeding or for preventing or treating malnutrition or other conditions or disease states. As background, it should be noted that human breast milk has a fatty acid profile comprising from about 0.15% to about 0.36% as DHA, from about 0.03% to about 0.13% as EPA, from about 0.30% to about 0.88% as AA, from about 0.22% to about 0.67% as DGLA, and from about 0.27% to about 1.04% as GLA. Thus, fatty acids such as AA, EPA and/or docosahexaenoic acid (DHA), produced in accordance with the present invention, can be used to alter, for example, the composition of infant formulas in order to better replicate the PUFA content of human breast milk or to alter the presence of PUFAs normally found in a non-human mammal's milk. In particular, a composition for use in a pharmacologic or food supplement, particularly a breast milk substitute or supplement, will preferably comprise one or more of AA, DGLA and GLA. More preferably, the oil will comprise from about 0.3 to 30% AA, from about 0.2 to 30% DGLA, and/or from about 0.2 to about 30% GLA.

Parenteral nutritional compositions comprising from about 2 to about 30 weight percent fatty acids calculated as triglycerides are encompassed by the present invention. The

preferred composition has about 1 to about 25 weight percent of the total PUFA composition as GLA (U.S. Pat. No. 5,196,198). Other vitamins, particularly fat-soluble vitamins such as vitamin A, D, E and L-carnitine can optionally be included. When desired, a preservative such as alpha-tocopherol may be added in an amount of about 0.1% by weight.

In addition, the ratios of AA, DGLA and GLA can be adapted for a particular given end use. When formulated as a breast milk supplement or substitute, a composition which comprises one or more of AA, DGLA and GLA will be provided in a ratio of about 1:19:30 to about 6:1:0.2, respectively. For example, the breast milk of animals can vary in ratios of AA:DGLA:GLA ranging from 1:19:30 to 6:1:0.2, which includes intermediate ratios which are preferably about 1:1:1, 1:2:1, 1:1:4. When produced together in a host cell, adjusting the rate and percent of conversion of a precursor substrate such as GLA and DGLA to AA can be used to precisely control the PUFA ratios. For example, a 5% to 10% conversion rate of DGLA to AA can be used to produce an AA to DGLA ratio of about 1:19, whereas a conversion rate of about 75% TO 80% can be used to produce an AA to DGLA ratio of about 6:1. Therefore, whether in a cell culture system or in a host animal, regulating the timing, extent and specificity of human Δ5-desaturase expression, as well as the expression of other desaturases and elongases, can be used to modulate PUFA levels and ratios. The PUFAs/acids produced in accordance with the present invention (e.g., AA and EPA) may then be combined with other PUFAs/acids (e.g., GLA) in the desired concentrations and ratios.

Additionally, PUFA produced in accordance with the present invention or host cells containing them may also be used as animal food supplements to alter an animal's tissue or milk fatty acid composition to one more desirable for human or animal consumption.

Pharmaceutical Compositions

The present invention also encompasses a pharmaceutical composition comprising one or more of the acids and/or resulting oils produced using the human Δ5-desaturase gene, in accordance with the methods described herein. More specifically, such a pharmaceutical composition may comprise one or more of the acids and/or oils as well as a standard, well-known, non-toxic pharmaceutically acceptable carrier, adjuvant or vehicle such as, for example, phosphate buffered saline, water, ethanol, polyols, vegetable oils, a wetting agent or an emulsion such as a water/oil emulsion. The composition may be in either a liquid or solid form. For example, the composition may be in the form of a tablet, capsule, ingestible liquid or powder, injectible, or topical ointment or cream. Proper fluidity can be maintained, for example, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. It may also be desirable to include isotonic agents, for example, sugars, sodium chloride and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening agents, flavoring agents and perfuming agents.

Suspensions, in addition to the active compounds, may comprise suspending agents such as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth or mixtures of these substances.

Solid dosage forms such as tablets and capsules can be prepared using techniques well known in the art. For example, PUFAs produced in accordance with the present invention can be tableted with conventional tablet bases such as lactose, sucrose, and cornstarch in combination with binders such as acacia, cornstarch or gelatin, disintegrating agents such as potato starch or alginic acid, and a lubricant such as stearic acid or magnesium stearate. Capsules can be prepared by incorporating these excipients into a gelatin capsule along with antioxidants and the relevant PUFA(s). The antioxidant and PUFA components should fit within the guidelines presented above.

For intravenous administration, the PUFAs produced in accordance with the present invention or derivatives thereof may be incorporated into commercial formulations such as Intralipids™. The typical normal adult plasma fatty acid profile comprises 6.64 to 9.46% of AA, 1.45 to 3.11% of DGLA, and 0.02 to 0.08% of GLA. These PUFAs or their metabolic precursors can be administered alone or in combination with other PUFAs in order to achieve a normal fatty acid profile in a patient. Where desired, the individual components of the formulations may be provided individually, in kit form, for single or multiple use. A typical dosage of a particular fatty acid is from 0.1 mg to 20 g (up to 100 g) daily and is preferably from 10 mg to 1, 2, 5 or 10 g daily.

Possible routes of administration of the pharmaceutical compositions of the present invention include, for example, enteral (e.g., oral and rectal) and parenteral. For example, a liquid preparation may be administered, for example, orally or rectally. Additionally, a homogenous mixture can be completely dispersed in water, admixed under sterile conditions with physiologically acceptable diluents, preservatives, buffers or propellants in order to form a spray or inhalant.

The route of administration will, of course, depend upon the desired effect. For example, if the composition is being utilized to treat rough, dry, or aging skin, to treat injured or burned skin, or to treat skin or hair affected by a disease or condition, it may perhaps be applied topically.

The dosage of the composition to be administered to the patient may be determined by one of ordinary skill in the art and depends upon various factors such as weight of the patient, age of the patient, immune status of the patient, etc.

With respect to form, the composition may be, for example, a solution, a dispersion, a suspension, an emulsion or a sterile powder which is then reconstituted.

The present invention also includes the treatment of various disorders by use of the pharmaceutical and/or nutritional compositions described herein. In particular, the compositions of the present invention may be used to treat restenosis after angioplasty. Furthermore, symptoms of inflammation, rheumatoid arthritis, asthma and psoriasis may also be treated with the compositions of the invention. Evidence also indicates that PUFAs may be involved in calcium metabolism; thus, the compositions of the present invention may, perhaps, be utilized in the treatment or prevention of osteoporosis and of kidney or urinary tract stones.

Additionally, the compositions of the present invention may also be used in the treatment of cancer. Malignant cells have been shown to have altered fatty acid compositions. Addition of fatty acids has been shown to slow their growth, cause cell death and increase their susceptibility to chemotherapeutic agents. Moreover, the compositions of the present invention may also be useful for treating cachexia associated with cancer.

The compositions of the present invention may also be used to treat diabetes (see U.S. Pat. No. 4,826,877 and Horrobin et al., *Am. J. Clin. Nutr.* Vol. 57 (Suppl.)

732S–737S). Altered fatty acid metabolism and composition have been demonstrated in diabetic animals.

Furthermore, the compositions of the present invention, comprising PUFAs produced either directly or indirectly through the use of the human Δ5-desaturase enzyme, may also be used in the treatment of eczema, in the reduction of blood pressure, and in the improvement of mathematics examination scores. Additionally, the compositions of the present invention may be used in inhibition of platelet aggregation, induction of vasodilation, reduction in cholesterol levels, inhibition of proliferation of vessel wall smooth muscle and fibrous tissue (Brenner et al., *Adv. Exp. Med. Biol.* Vol. 83, p.85–101, 1976), reduction or prevention of gastrointestinal bleeding and other side effects of non-steroidal anti-inflammatory drugs (see U.S. Pat. No. 4,666, 701), prevention or treatment of endometriosis and premenstrual syndrome (see U.S. Pat. No. 4,758,592), and treatment of myalgic encephalomyelitis and chronic fatigue after viral infections (see U.S. Pat. No. 5,116,871).

Further uses of the compositions of the present invention include use in the treatment of AIDS, multiple sclerosis, and inflammatory skin disorders, as well as for maintenance of general health.

Additionally, the composition of the present invention may be utilized for cosmetic purposes. It may be added to pre-existing cosmetic compositions such that a mixture is formed or may be used as a sole composition.

Veterinary Applications

It should be noted that the above-described pharmaceutical and nutritional compositions may be utilized in connection with animals (i.e., domestic or non-domestic), as well as humans, as animals experience many of the same needs and conditions as humans. For example, the oil or acids of the present invention may be utilized in animal feed supplements, animal feed substitutes, animal vitamins or in animal topical ointments.

The present invention may be illustrated by the use of the following non-limiting examples:

EXAMPLE I

Human Desaturase Gene Sequences

As described in International Application PCT/US98/07422 (herein incorporated in its entirety by reference), the putative human desaturase gene sequences involved in long chain polyunsaturated fatty acid biosynthesis were isolated based on homology between the human cDNA sequences and *Mortierella alpina* desaturase gene sequences. The three conserved "histidine boxes" known to be conserved among membrane-bound desaturases were found. As with other membrane-bound desaturases, the final HXXHH histidine box motif was found to be QXXHH. The amino acid sequence of the putative human desaturases exhibited homology to *M. alpina* Δ5-, Δ6-, Δ9-, and Δ12-desaturases.

The *M. alpina* Δ5-desaturase and Δ6-desaturase cDNA sequences were used to search the LifeSeq database of Incyte Pharmaceuticals, Inc., Palo Alto, Calif. The Δ5-desaturase sequence was divided into fragments: 1) amino acid no. 1–150, 2) amino acid no. 151–300, and 3) amino acid no. 301–446. The Δ6 desaturase sequence was divided into three fragments: 1) amino acid no. 1–150, 2) amino acid no. 151–300, and 3) amino acid no. 301–457. These polypeptide fragments were searched against the database using the "tblastn" algorithm. This algorithm compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands).

The polypeptide fragments 2 and 3 of *M. alpina* Δ5- and Δ6-desaturases have homologies with the CloneID sequences as outlined in FIG. 1. The CloneID represents an individual sequence from the Incyte LifeSeq database. After the "tblastn" results had been reviewed, Clone Information was searched with the default settings of Stringency of >=50, and Productscore <=100 for different CloneID numbers. The Clone Information Results displayed the information including the ClusterID, CloneID, Library, HitID, and Hit Description. When selected, the ClusterID number displayed the clone information of all the clones that belong in that ClusterID. The Assemble command assembled all of the CloneID which comprise the ClusterID. The following default setting were used for GCG (Genetics Computer Group, University of Wisconsin Biotechnology Center, Madison, Wis.) Assembly:

Word Size: 7; Minimum Overlap: 14; Stringency: 0.8; Minimum Identity: 14; Maximum Gap: 10; Gap Weight: 8; and Length Weight: 2.

GCG Assembly Results displayed the contigs generated on the basis of sequence information within the CloneID. A contig is an alignment of DNA sequences based on areas of homology among these sequences. A new sequence (consensus sequence) was generated based on the aligned DNA sequence within a contig. The contig. containing the CloneID was identified, and the ambiguous sites of the consensus sequence were edited based on the alignment of the CloneIDs (see FIGS. 2–6) to generate the best possible sequence. The procedure was repeated for all six CloneID listed in FIG. 1. This produced five unique contigs. The edited consensus sequences of the 5 contigs were imported into the Sequencher software program (Gene Codes Corporation, Ann Arbor, Mich.). These consensus sequences were assembled. The contig 2511785 overlaps with contig 3506132, and this new contig was called 2535 (FIG. 7). The contigs from the Sequencher program were copied into the Sequence Analysis software package of GCG.

Each contig was translated in all six reading frames into protein sequences. The *M. alpina* Δ5-desaturase (Ma29) and Δ6-desaturase (Ma524) sequences were compared with each of the translated contigs using the FastA search (a Pearson and Lipman search for similarity between a query sequence and a group of sequences of the same type (nucleic acid or protein)). Homology among these sequences suggest the open reading frames of each contig as underlined in FIGS. 3, 5, and 7. The homology among the *M. alpina* Δ5- and Δ6-desaturase sequences to contigs 2535 and 3854933 were utilized to create the final contig called 253538a (see FIG. 8). FIG. 9 is the FastA match of the translated sequences of the final contig 253538a and Ma29, and FIG. 10 is the FastA match of the translated sequences of the final contig 253538a and Ma524.

Although the open reading frame was generated by merging the two contigs, the contig 2535 shows that there is a unique sequence in the beginning of this contig which does not match with the contig 3854933. Therefore, it is possible that these contigs were generated from independent desaturase-like human genes.

The contig 253538a contains an open reading frame encoding 432 amino acid (FIG. 8, underlined). It starts with Gln (CAG) and ends with the stop codon (TGA) (both in bold). The contig 253538a aligns with both *M. alpina* Δ5- and Δ6-desaturase sequences, suggesting that it could be either of the desaturases, as well as other known desaturases which share homology with each other. The individual contigs listed in FIG. 1, as well as the intermediate contig 2535 and the final contig 253538a can be utilized to isolate the complete genes for human desaturases.

Determination of Human Δ5-Desaturase Gene Sequence

Primers RO384 and RO388 were designed based on the 5' and 3' sequences, respectively, of contig 2535. The human monocyte cDNA library (Clontech, Palo Alto, Calif.) was amplified with the vector primer RO329 (5'-CAG ACC AAC TGG TAA TGG TAG-3') SEQ ID NO:49) and RO384 (5'-TCA GGC CCA AGC TGG ATG GCT GCA ACA TG-3'), (SEQ ID NO:50) and also with the vector primer RO328 (5'-CTC CTG GAG CCC GTC AGT ATC-3') (SEQ ID NO:51) and RO388 (5'-ATG GTG GGG AAG AGG TGG TGC TCA ATC TG-3') (SEQ ID NO:52). Polymerase Chain Reaction (PCR) was carried out in a 100 μl volume containing: 1 μl of human monocyte cDNA library, 10 pM each primer, 10 μl of 10×buffer and 1.0 U of Taq Polymerase. Thermocycler conditions in Perkin Elmer 9600 were as follows: 94° C. for 2 mins, then 30 cycles of 94° C. for 1 min., 58° C. for 2 mins. and 72° C. for 3 mins. PCR was followed by an additional extension at 72° C. for 7 minutes.

The PCR amplified mixture was run on a gel, and the amplified fragments were gel purified. The isolated fragment from PCR amplification with RO329 and RO384 was approximately 900 bp, and that from PCR amplification with RO328 and RO388 was approximately 650 bp. These isolated fragments were filled-in using T4 DNA polymerase, and the filled-in fragments were cloned into the PCR-Blunt vector (Invitrogen Corp., Carlsbad, Calif.). The clone of RO329/RO384 amplified fragment was designated as pRAE-7, and the clone of RO328/RO388 amplified fragment was designated as pRAE-8. Both ends of the clones were sequenced using ABI 373 DNA Sequencer (Applied Biosystems, Foster City, Calif.) and assembled using the Sequencher program (a sequence analysis program, Gene Codes Corporation, Ann Arbor, Mich.). This assembly of the sequences revealed that the two clones contained different sizes of the same gene (FIG. **14**). The complete sequence of the pRAE-7 gene was compiled (FIG. **15**) and searched against the known sequences in the public database.

The FastA algorithm is a Pearson and Lipman search for similarity between a query sequence and a group of sequences of the same type (nucleic acid or protein). The pRAE-7 gene sequence was translated in six reading frames, and using this method, the Swissprot database (Genetics Computer Group (GCG) (Madison, Wis.) was searched. The gene in pRAE-7 was identified as a putative human desaturase based on its homology to known desaturases. The Swissprot database search produced matches against the omega-3 fatty acid desaturase from mung bean (23.4% identity in 303 AA overlap), linoleoyl-CoA desaturase from Synechocystis sp. (24.3% identity in 280 AA overlap), omega-6 fatty acid desaturase from soybean (19.7% identity in 284 AA overlap), and acyl-CoA desaturase 1 from *Saccharomyces cerevisiae* (21.6% identity in 134 AA overlap). The FastA search against the *M. alpina* desaturases produced matches against the Δ6-(31.9% identity in 285 AA overlap), the Δ5-(28.4% identity in 292 AA overlap), and the Δ12-(23.0% identity in 274 AA overlap) desaturases. The matched sequence alignment of the putative human desaturase gene in pRAE-7 against *M. alpina* Δ5-desaturase (Ma29), *M. alpina* Δ6-desaturase (Ma524) as well as to the contigs 2535 and 38 are displayed in FIGS. **16**, **17**, **18**, and **19** respectively.

The contigs 2535, 38, and 253538a were generated based on assemblies of various sequences as well as their homologies against the known desaturases. However, upon examining FIGS. **18** and **19**, it can be concluded that the contigs are merely indications as to what the sequences of the human desaturases might possibly be.

The 5' end of the gene, the ATG (Methionine), is necessary for expressing the human desaturase in yeast. FIGS. **16** and **17** show that pRAE-7 is probably just the last ⅔ of a desaturase gene. Several of the omega-3 and omega-6 fatty acid desaturases, as well as the linoleoyl-CoA desaturase mentioned above, are smaller than the *M. alpina* Δ5- and Δ6-desaturases, ranging in sizes of 359–380 amino acids. It was concluded from all of the sequences evaluated thus far that the isolated gene probably needed anywhere from 180–480 bp (60–160 amino acids) of additional 5' sequence for expressing a complete enzyme.

In order to extend the 5' sequence of the human desaturase gene, the Marathon cDNA Amplification Kit (Clontech, Palo Alto, Calif.) was used to screen the human liver marathon ready cDNA (Clontech). The rapid amplification of cDNA ends (RACE) reactions are efficient for both 5' and 3' long-distance PCR. Following the 5' RACE protocol outlined in the kit, the primers RO430 (5'-GTG GCT GTT GTT ATT GGT GAA GAT AGG CAT C-3') (designed based on the pRAE-7 gene 3' sequence, downstream of the TAA (stop)) and the marathon adaptor primer (AP1) from the kit, were used to generate three PCR amplified products, which were designated A, B, and C. The fragment sizes were approximately 1.5 Kb, 1.4 Kb, 1.2 Kb, respectively. The fragments were filled-in with T4 DNA polymerase, and cloned into the pCR-blunt vector. A total of twenty-two clones were generated and sequenced. Using the FastA algorithm, the sequences were searched against the GenEMBL database of GCG.

Many of the sequences had a great homology to the human DNA sequence with the GenBank accession number of AC004228. This DNA sequence is described as: Sequencing in Progress, *Homo sapiens* Chromosome 11q12pac pDJ519o3; HTGS phase 1,18 unordered pieces. The 18 contigs were recorded in an arbitrary fashion. Using this sequence information and the information from the assembled sequences of the clones, the clones were categorized into five groups.

All of the clones have the same sequence downstream of the BamHI site (see FIG. **12**, underlined). But each group represents a different 5' sequence, with a total of 10 clones being too short to be the full length gene. Group 1, represented by clone A-1, is comprised of 5 clones which have homology to cytochrome b5 gene (FIG. **20**). A translational start codon, ATG, is not present in clone A-1; however, as can be seen in FIG. **21**, there is an ATG (underlined) present in the ac004228 sequence 17 bp upstream of the strong area of homology between A-1 and ac004228. Starting from the strong area of homology, A-1 has an open reading frame of 1318 bp. However, starting from the ATG, the open reading frame is 1335 bp. Group 2, represented by clone 3-5, is comprised of 3 clones which have an ATG within an NcoI site, but four translational stop codons between the ATG and the BamHI site (FIG. **22**, the NcoI, BamHI sites are in bold, and the four termination codons are underlined). Group 3 is comprised of one clone, A-10, which has an ATG 135 bp upstream of the BamHI site, giving an open reading frame of 1267 bp (FIG. **23**). Group 4 is comprised of 2 clones, represented by clone A-16, which does not have an ATG; however, upstream of where the sequence aligns with ac004228, there is an ATG (FIG. **24**, underlined). The open reading frame of this group is 1347 bp. Group 5 is comprised of one clone which does not have an ATG. However, this clone matches the ac004228 sequence even upstream of the BamHI site (FIG. **25**).

As illustrated in FIG. **26**, many of the clones from the five groups are represented in order with the ac004228 sequence.

There appeared to be a high level of splicing, with the sequence downstream of the BamHI site (in bold) acting as the common anchor for the various 5' exons. All of the potential start sites are also in bold, and the sequences found within the clones have been underlined.

The A-1 sequence was used to search the LifeSeq database of Incyte Pharmaceuticals, Inc., Palo Alto, Calif., to see if its latest version would also have sequences with homology to our desaturase gene sequence. Two contigs were generated in this search, contig 3381584 and contig 2153526. The human desaturase gene sequence was initially compiled based on sequences from Group I clones and ac004228. However, FIG. **12** represents the actual DNA sequence of the isolated gene. The Incyte contigs were used to confirm this sequence (see FIGS. **27** and **28**). The human desaturase translated sequence, consisting of 445 amino acids (FIG. **13**), was also matched with the original contigs 253538a and 38. These alignments are shown in FIGS. **29** and **30**, respectively.

The FastA search of the human desaturase gene against the Swissprot database produced matches against the omega-3 fatty acid desaturase from mung bean (22.4% identity in 381 AA overlap), linoleoyl-CoA desaturase from Synechocystis Sp. (24.5% identity in 335 AA overlap), omega-6 fatty acid desaturase from soybean (20.3% identity in 290 AA overlap), and acyl-CoA desaturase 1 from *Saccharomyces cerevisiae* (21.4% identity in 168 AA overlap). The FastA search against *M. alpina* desaturases produced matches against the Δ6-(30.5% identity in 455 AA overlap), Δ5-(27.5% identity in 455 AA overlap), and Δ12-desaturases (22.5% identity in 382 AA overlap). The FastA match of the human desaturase translated sequence against the ma524 (*M. alpina* Δ6-desaturase) and ma29 (*M. alpina* Δ5-desaturase) sequences are shown in FIGS. **31** and **32**, respectively.

EXAMPLE II

Construction of Clones

New clones were generated based on clones from three of the Groups mentioned above, clones A-1, A-10, and A-16. Two primers which were modified with 5' phosphate, RO526 (5'-CAT GGC CCC CGA CCC GGT GG-3') (SEQ ID NO:54) and RO527 (5'-GCG GCC ACC GGG TCG GGG GC-3') (SEQ ID NO:55), were annealed together to form an adaptor. This adaptor which has NcoI and BsaI overhangs, were ligated with the A-1 clone, which had been cut with BsaI/HindIII and gel purified, for 15 min at room temperature. The pYX242(NcoI/HindIII) vector (Novagen, Madison, Wis.) was added to this ligation mixture and allowed to incubate at room temperature for an additional 45 min. This produced a clone designated as pRAE-28-5. (Plasmid pRAE-28-5 was deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209 on Dec. 22, 1998, under the terms of the Budapest Treaty, and was accorded ATCC number 203557.)

The A-10 clone was PCR amplified with RO512 (5'-GAT TGG GTG CCA TGG GGA TGC GGG ATG AAA AGG C-3') (SEQ ID NO:56) and RO5 (5'-GAA ACA GCT ATG ACC ATG-3') (SEQ ID NO:57), the amplified product was cut with NcoI and HindIII and gel purified, and the purified fragment was cloned into pYX242 (NcoI/HindIII). This new clone was designated as pRAE-26-1.

The A-10 clone was also PCR amplified with RO580 (5'-TCC TS TGC GAA TTC ACC ATG AAA AGG CGG GAG AGA G-3') (SEQ ID NO:58) and RO5, the amplified product was cut with NcoI and HindIII and gel purified, and the purified fragment was cloned into pYX242 (NcoI/HindIII). This new clone was designated as pRAE-33.

Two primers which were modified with 5' phosphate, RO578 (5'-CAT GGC TAG GAG AGG CAG CGC AGC CGC GTC TGG AC-3') (SEQ ID NO:59) and RO579 (5'-CTA GGT CCA GAC GCG GCT GCG CTG CCT CTC CTA GC-3') (SEQ ID NO:60), were annealed together to form an adaptor. This adaptor which has NcoI and BlnI overhangs, were ligated with the A-16 clone, which had been cut with BlnI/HindIII and gel purified, for 15 min at room temperature. The pYX242 (NcoI/HindIII) vector was added to this ligation mixture and allowed to incubate at room temperature for an additional 45 min. This produced a clone designated as pRAE-35.

EXAMPLE III

Expression of Human Δ5-Desaturase

The constructs pRAE-26-1, pRAE-28-5, pRAE-33, and pRAE-35 were transformed into *S. cerevisiae* 334 and screened for desaturase activity. The substrates DGLA (20:3n-6), OA (18:1n-9), AA (20:4n-6), and LA (18:2n-6) were used to determine the activity of the expressed gene from constructs pRAE-26-1 and pRAE-28-5. Only the substrate DGLA was used to determine the activity of the expressed gene from all of the constructs. The negative control strain was *S. cerevisiae* 334 containing the unaltered pYX242 vector. The cultures were grown for 48 hours at 30° C., in selective media (Ausubel et al., *Short Protocols in Molecular Biology*, Ch. 13, P. 3–5 (1992)), in the presence of a particular substrate. Lipid fractions of each culture were extracted for analysis. The desaturase activity results are provided in FIGS. **33** and **34**.

All of the values in FIG. **33** are the average of two separate samples per strain, tested in the same run. The substrate, as well as the fatty acid it was converted to, is shown in bold. The expressed gene in the strain 334 (pRAE-28-5) is a Δ5-desaturase. It converted the substrate DGLA to a higher percent of AA than the control strain 334 (pYX242), 0.127% vs. 0.062%, respectively. The percent of AA present in the cultures of strains 334 (pRAE-26-1), 334 (pRAE-33), and 334 (pRAE-35) are comparable to that of the control strain (0.075%, 0.062%, and 0.063%, respectively). Therefore, it can be concluded that the cyt b5 sequence containing gene in the construct pRAE-28-5 expresses an active human Δ5-desaturase; whereas, the other variations of the gene do not.

The activity of the human Δ5-desaturase was further confirmed in the experiment outlined in FIG. **34**. Included in this figure are the fatty acid profiles of the strains 334 (pRAE-28-5), 334 (pRAE-26-1), and the control strain 334 (pYX242) when DGLA (20:3n-6), OA (18:1n-9), AA (20:4n-6), or LA (18:2n-6) was used as the substrate, as well as when no substrate was added. Again, the strain 334 (pRAE-28-5) expressed an active human Δ5-desaturase, converting DGLA to AA at a higher percent than the control strain, 0.106% vs. 0.065%, respectively. The strain 334 (pRAE-26-1) had about the same amount of AA (0.06%) as the control. The conversion of the substrate OA to LA was not detected, confirming that the strains do not have a Δ12-desaturase activity. The conversion of the substrate AA to eicosapentaenoic acid (EPA, 20:5n-3) was detected, but at a very low level equal to that of the control strain, confirming that the strains do not have a Δ17-desaturase activity. The conversion of the substrate LA to GLA was detected, but again at a very low level equal to the control strain, confirming that the strains do not have a Δ6-desaturase activity.

The present sequence (FIG. 12) differs from the Genbank sequence g3169158 of the LifeSeq database with respect to two positions. In particular, with respect to the nucleotide sequence of sequence g3169158, position 1082 is an adenosine; however, in the present sequence position 1082 is a thymine (see FIG. 12). Furthermore, position 1229 of sequence g3169158 is an adenine whereas in the present sequence position 1229 is a guanine. In terms of an amino acid sequence comparison, position 361 of the present sequence is a leucine (see FIG. 13), and position 361 of sequence g3169158 is a glutamine. Furthermore, position 410 of the present sequence is an arginine, whereas position 410 of sequence g3169158 is a histidine. Additionally, sequence g3169158 is described, in the database, as a "hypothetical protein" which "exhibits similarity to motifs found in delta 6 desaturase, a hypothetical cytochrome b5 containing fusion protein." However, as demonstrated in the above example, the protein encoded by the sequence in FIG. 12 is a human Δ5-desaturase, not a Δ6-desaturase.

EXAMPLE IV

Expression of Human Δ5-Desaturase in Insect Cells

Insect cells were used as another eukaryotic host for expression of the human Δ5-desaturase. The baculovirus expression system involves the use of insect cells to express a gene, in this case, the human Δ5-desaturase, which has been cloned into a baculovirus expression vector. Insect cells are known to have no endogenous PUFA desaturase activities. Therefore, this system is suitable for expression and characterization of the recombinant desaturases.

The fragment containing the human Δ5-desaturase gene (pRAE-28-5, see EXAMPLE II) was PCR amplified using Expand High Fidelity PCR System (Boehringer Mannheim Corp., Indianapolis, Ind.) and a set of primers containing appropriate restriction sites. The upstream primer designated RO676 (5'-ATA CGT GAA TTC GCC GCC ACC ATG GCC CCC GAC CCG GTG-3') (SEQ ID NO:49) corresponded to the sense strand of Δ5 cDNA and contained an EcoRI site 5' upstream of the ATG. The downstream primer RO677 (5'-TAT CCG CTC GAG TTA TTG GTG AAG ATA GGC ATC TAG-3') (SEQ ID NO:48) corresponded to the antisense strand at the 3' end of the Δ5 cDNA, and included an XhoI site immediately downstream of the translational termination codon. The PRC reaction, in a final volume of 100 μl, was carried out as follows: 5 mins denaturation at 94° C., then 45 seconds at 94° C., 45 seconds at 55° C. and 2 min at 72° C. for 30 cycles, and 7 mins. extension at 72° C. at the end of the amplification. The human Δ5 PCR amplified product was analyzed by agarose-gel electrophoresis, gel purified, digested with EcoRI and XhoI, and then ligated into pFastBac1 baculovirus donor plasmid (Gibco-BRL, Gaithersburg, Md.) which was restricted with the same enzymes. The respective baculovirus clone was designated as pJPBh4 for the human Δ5-desaturase. This pFastBac1 vector contains an expression cassette which has a polyhedrin promoter, a SV40 polyadenylation signal, and a gentamycin resistance marker.

The initial transformation was done in XL1 blue cells (Invitrogen, Carlsbad, Calif.). Positive clones were then transformed into *E. coli* DH10Bac (Gibco-BRL, Gaithersburg, Md.) which contains the baculovirus genome. The positive clones were selected by blue white screening in which white colonies contain the recombinant bacmid. White colonies were then selected for bacmid DNA isolation. DNA was isolated using a Qiagen plasmid isolation kit (Qiagen, Inc., Valencia, Calif.), specific for DNA over 135 kb long. The recombinant bacmid DNA was analyzed on a 0.6% agarose gel to confirm the presence of the high molecular weight DNA. PCR analysis, using pUC/M13 primers (forward 5'-TGT AAA ACG ACG GCC AGT-3' and reverse 5'-GAA ACA GCT ATG ACC ATG-3') was also performed to confirm the correct insert size for the desaturase cDNA within the bacmid.

The Sf9 insect cells (*Spodoptera frugiperda*) were used for the recombinant bacmid DNA transfection. These cells were grown in serum free media (Gibco-BRL, Gaithersburg, Md.). Transfection was carried out according to the Cell-FECTIN Sf900 protocol (Gibco-BRL, Gaithersburg, Md.). The recombinant virus was recovered by collecting the supernatant at 72 hours post-transfection. A plaque assay was performed on the supernatant to determine the titer of recovered recombinant virion particles. A recombinant viral stock was made for the expression studies. All infections with the recombinant virus were done during the mid-logarithmic growth phase of the Sf9's and infected at 5 MOI (Multiplicity of Infection). To analyze the activity of the expressed human Δ5-desaturase gene, the Sf9m cells were plated at a concentration of 1×10^6 cells/well in a 6-well tissue culture plate and infected with 100 μl of the virus stock (approximately 5 MOI). The substrate, dihomo-gamma-linolenic acid (DGLA, C20:3n-6). was supplemented at the time of infection, at a concentration of 100 μM. A mock infected Sf9, as well as cells infected with a recombinant virus containing the GusA reporter gene, were used as negative controls in each experiment. The medium was collected 48 hours post infection and saved. The cells were collected and submitted for lipid analysis.

For fatty acid analysis, cell pellets were vortexed with 6 ml of methanol, followed by the addition of 12 ml of chloroform and tridecanoin (as internal standard). The mixtures were incubated for at least one hour at room temperature or at 4° C. overnight. The chloroform layer was extracted and filtered through a Whatman filter with one gram of anhydrous sodium sulfate to remove particulates and residual water. The organic solvents were evaporated at 40° C. under a stream of nitrogen. The extracted lipids were derivatized to fatty acid methyl esters (FAME) for gas chromatography analysis (GC) by adding 2 ml of 0.5 N potassium hydroxide in methanol to a closed tube. The samples were heated at 95 to 100° C. for 30 minutes and cooled to room temperature. Approximately 2 ml of the 14% boron trifluoride in methanol was added and the heating repeated. After the extracted lipid mixture cooled, 2 ml of water and 1 ml of hexane were added to extract the FAME for GC analysis. The percent conversion was calculated by dividing the product produced by the sum of (the product produced and the substrate) and then multiplying by 100.

The fatty acid synthesis in insect cells infected with recombinant virus containing the human Δ5 cDNA is summarized in Table 1. The conversion of the added substrate, DGLA (C20:3n-6), to arachidonic acid (AA, 20:4n-6) was monitored. The quantity of arachidonic acid (AA, 20:4n-6) produced by the human Δ5-desaturase was 9.67% of the total fatty acid versus the control which did not produce any AA. This resulted in a 29.6% conversion of DGLA to AA.

These data indicate that the human Δ5-desaturase can be expressed in another eukaryotic host (insect cells) in a biologically active form as demonstrated by the production of AA.

TABLE 1

Fatty Acid	Human Δ5	Control
18:1n-9	19.15	19.99
18:3n-6	2.43	5.18
*20:3n-6	22.95	30.00
20:4n-6 (29.6%)	9.67	ND
22:1n-9	0.11	0.25

*indicates substrate added
ND indicates None Detected

Nutritional Compositions

The PUFAs described in the Detailed Description may be utilized in various nutritional supplements, infant formulations, nutritional substitutes and other nutritional solutions.

I. Infant Formulations

A. Isomil® Soy Formula with Iron

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cows milk. A feeding for patients with disorders for which lactose should be avoided: lactase deficiency, lactose intolerance and galactosemia.

Features

- Soy protein isolate to avoid symptoms of cowls-milk-protein allergy or sensitivity.
- Lactose-free formulation to avoid lactose-associated diarrhea.
- Low osmolality (240 mOs/kg water) to reduce risk of osmotic diarrhea.
- Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.
- 1.8 mg of Iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- Recommended levels of vitamins and minerals.
- Vegetable oils to provide recommended levels of essential fatty acids.
- Milk-white color, milk-like consistency and pleasant aroma.

Ingredients: (Pareve) 85% water, 4.9% corn syrup, 2.6% sugar (sucrose), 2.1% soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11% calcium phosphate tribasic, potassium citrate, potassium phosphate monobasic, potassium chloride, mono- and disglycerides, soy lecithin, carrageenan, ascorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

B. Isomil® DF Soy Formula for Diarrhea

Usage: As a short-term feeding for the dietary management of diarrhea in infants and toddlers.

Features

- First infant formula to contain added dietary fiber from soy fiber specifically for diarrhea management.
- Clinically shown to reduce the duration of loose, watery stools during mild to severe diarrhea in infants.
- Nutritionally complete to meet the nutritional needs of the infant.
- Soy protein isolate with added L-methionine meets or exceeds an infant's requirement for all essential amino acids.
- Lactose-free formulation to avoid lactose-associated diarrhea.
- Low osmolality (240 mOsm/kg water) to reduce the risk of osmotic diarrhea.
- Dual carbohydrates (corn syrup and sucrose) designed to enhance carbohydrate absorption and reduce the risk of exceeding the absorptive capacity of the damaged gut.
- Meets or exceeds the vitamin and mineral levels recommended by the Committee on Nutrition of the American Academy of Pediatrics and required by the Infant Formula Act.
- 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- Vegetable oils to provide recommended levels of essential fatty acids.

Ingredients: (Pareve) 86% water, 4.8% corn syrup, 2.5% sugar (sucrose), 2.1% soy oil, 2.0% soy protein isolate, 1.4% coconut oil, 0.77% soy fiber, 0.12% calcium citrate, 0.11% calcium phosphate tribasic, 0.10% potassium citrate, potassium chloride, potassium phosphate monobasic, mono and diglycerides, soy lecithin, carrageenan, magnesium chloride, ascorbic acid, L-methionine, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

C. Isomil® SF Sucrose-Free Soy Formula with Iron

Usage: As a beverage for infants, children and adults with an allergy or sensitivity to cow's-milk protein or an intolerance to sucrose. A feeding for patients with disorders for which lactose and sucrose should be avoided.

Features

- Soy protein isolate to avoid symptoms of cow's-milk-protein allergy or sensitivity.
- Lactose-free formulation to avoid lactose-associated diarrhea (carbohydrate source is Polycose® Glucose Polymers).
- Sucrose free for the patient who cannot tolerate sucrose.
- Low osmolality (180 mOsm/kg water) to reduce risk of osmotic diarrhea.
- 1.8 mg of iron (as ferrous sulfate) per 100 Calories to help prevent iron deficiency.
- Recommended levels of vitamins and minerals.
- Vegetable oils to provide recommended levels of essential fatty acids.
- Milk-white color, milk-like consistency and pleasant aroma.

Ingredients: (Pareve) 75% water, 11.8% hydrolized cornstarch, 4.1% soy oil, 4.1% soy protein isolate, 2.8% coconut oil, 1.0% modified cornstarch, 0.38% calcium phosphate tribasic, 0.17% potassium citrate, 0.13% potassium chloride, mono- and diglycerides, soy lecithin, magnesium chloride, abscorbic acid, L-methionine, calcium carbonate, sodium chloride, choline chloride, carrageenan, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

D. Isomil® 20 Soy Formula with Iron Ready to Feed, 20 Cal/fl oz.

Usage: When a soy feeding is desired.

Ingredients: (Pareve) 85% water, 4.9% corn syrup, 2.6% sugar(sucrose), 2.1% soy oil, 1.9% soy protein isolate, 1.4% coconut oil, 0.15% calcium citrate, 0.11% calcium phosphate tribasic, potassium citrate, potassium phosphate monobasic, potassium chloride, mono- and diglycerides, soy lecithin, carrageenan, abscorbic acid, L-methionine, magnesium chloride, potassium phosphate dibasic, sodium chloride, choline chloride, taurine, ferrous sulfate, m-inositol, alpha-tocopheryl acetate, zinc sulfate, L-carnitine, niacinamide, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, potassium iodide, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

E. Similac® Infant Formula

Usage: When an infant formula is needed: if the decision is made to discontinue breastfeeding before age 1 year, if a supplement to breastfeeding is needed or as a routine feeding if breastfeeding is not adopted.

Features

- Protein of appropriate quality and quantity for good growth; heat-denatured, which reduces the risk of milk-associated enteric blood loss.
- Fat from a blend of vegetable oils (doubly homogenized), providing essential linoleic acid that is easily absorbed.
- Carbohydrate as lactose in proportion similar to that of human milk.
- Low renal solute load to minimize stress on developing organs.

Powder, Concentrated Liquid and Ready To Feed forms.

Ingredients: (-D) Water, nonfat milk, lactose, soy oil, coconut oil, mono- and diglycerides, soy lecithin, abscorbic acid, carrageenan, choline chloride, taurine, m-inositol, alpha-tocopheryl acetate, zinc sulfate, niacinamide, ferrous sulfate, calcium pantothenate, cupric sulfate, vitamin A palmitate, thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

F. Similac® NeoCare Premature Infant Formula with Iron

Usage: For premature infants' special nutritional needs after hospital discharge. Similac NeoCare is a nutritionally complete formula developed to provide premature infants with extra calories, protein, vitamins and minerals needed to promote catch-up growth and support development.

Features

- Reduces the need for caloric and vitamin supplementation. More calories (22 Cal/fl oz) than standard term formulas (20 Cal/fl oz).
- Highly absorbed fat blend, with medium-chain triglycerides (MCT oil) to help meet the special digestive needs of premature infants.
- Higher levels of protein, vitamins and minerals per 100 calories to extend the nutritional support initiated in-hospital.
- More calcium and phosphorus for improved bone mineralization.
- Ingredients: -D Corn syrup solids, nonfat milk, lactose, whey protein concentrate, soy oil, high-oleic safflower oil, fractionated coconut oil (medium chain triglycerides), coconut oil, potassium citrate, calcium phosphate tribasic, calcium carbonate, ascorbic acid, magnesium chloride, potassium chloride, sodium chloride, taurine, ferrous sulfate, m-inositol, choline chloride, ascorbyl palmitate, L-carnitine, alpha-tocopheryl acetate, zinc sulfate, niacinamide, mixed tocopherols, sodium citrate, calcium pantothenate, cupric sulfate, thiamine chloride hydrochloride, vitamin A palmitate, beta carotene, riboflavin, pyridoxine hydrochloride, folic acid, manganese sulfate, phylloquinone, biotin, sodium selenite, vitamin D3 and cyanocobalamin.

G. Similac Natural Care Low-Iron Human Milk Fortifier Ready to Use, 24 Cal/fl oz.

Usage: Designed to be mixed with human milk or to be fed alternatively with human milk to low-birth-weight infants.

Ingredients: -D Water, nonfat milk, hydrolyzed cornstarch, lactose, fractionated coconut oil (medium-chain triglycerides), whey protein concentrate, soy oil, coconut oil, calcium phosphate tribasic, potassium citrate, magnesium chloride, sodium citrate, ascorbic acid, calcium carbonate, mono and diglycerides, soy lecithin, carrageenan, choline chloride, m-inositol, taurine, niacinamide, L-carnitine, alpha tocopheryl acetate, zinc sulfate, potassium chloride, calcium pantothenate, ferrous sulfate, cupric sulfate, riboflavin, vitamin A palmitate, thiamine chloride hydrochloride, pyridoxine hydrochloride, biotin, folic acid, manganese sulfate, phylloquinone, vitamin D3, sodium selenite and cyanocobalamin.

Various PUFAs of this invention can be substituted and/or added to the infant formulae described above and to other infant formulae known to those in the art.

II. Nutritional Formulations

A. ENSURE®

Usage: ENSURE is a low-residue liquid food designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets. Although it is primarily an oral supplement, it can be fed by tube.

Patient Conditions

- For patients on modified diets
- For elderly patients at nutrition risk
- For patients with involuntary weight loss
- For patients recovering from illness or surgery
- For patients who need a low-residue diet

Ingredients: -D Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Sodium Molybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate.

B. Ensure® Bars

Usage: ENSURE BARS are complete, balanced nutrition for supplemental use between or with meals. They provide a delicious, nutrient-rich alternative to other snacks. ENSURE BARS contain <1 g lactose/bar, and Chocolate Fudge Brownie flavor is gluten-free. (Honey Graham Crunch flavor contains gluten.)

Patient Conditions

For patients who need extra calories, protein, vitamins and minerals.

Especially useful for people who do not take in enough calories and nutrients.

For people who have the ability to chew and swallow

Not to be used by anyone with a peanut allergy or any type of allergy to nuts.

Ingredients: Honey Graham Crunch—High-Fructose Corn Syrup, Soy Protein Isolate, Brown Sugar, Honey, Maltodextrin (Corn), Crisp Rice (Milled Rice, Sugar [Sucrose], Salt [Sodium Chloride] and Malt), Oat Bran, Partially Hydrogenated Cottonseed and Soy Oils, Soy Polysaccharide, Glycerine, Whey Protein Concentrate, Polydextrose, Fructose, Calcium Caseinate, Cocoa Powder, Artificial Flavors, Canola Oil, High-Oleic Safflower Oil, Nonfat Dry Milk, Whey Powder, Soy Lecithin and Corn Oil. Manufactured in a facility that processes nuts.

Vitamins and Minerals: Calcium Phosphate Tribasic, Potassium Phosphate Dibasic, Magnesium Oxide, Salt (Sodium Chloride), Potassium Chloride, Ascorbic Acid, Ferric Orthophosphate, Alpha-Tocopheryl Acetate, Niacinamide, Zinc Oxide, Calcium Pantothenate, Copper Gluconate, Manganese Sulfate, Riboflavin, Beta Carotene, Pyridoxine Hydrochloride, Thiamine Mononitrate, Folic Acid, Biotin, Chromium Chloride, Potassium Iodide, Sodium Selenate, Sodium Molybdate, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein: Honey Graham Crunch—The protein source is a blend of soy protein isolate and milk proteins.

Soy protein isolate	74%
Milk proteins	26%

Fat: Honey Graham Crunch—The fat source is a blend of partially hydrogenated cottonseed and soybean, canola, high oleic safflower, oils, and soy lecithin.

Partially hydrogenated cottonseed and soybean oil	76%
Canola oil	8%
High-oleic safflower oil	8%
Corn oil	4%
Soy lecithin	4%

Carbohydrate: Honey Graham Crunch—The carbohydrate source is a combination of high-fructose corn syrup, brown sugar, maltodextrin, honey, crisp rice, glycerine, soy polysaccharide, and oat bran.

High-fructose corn syrup	24%
Brown sugar	21%
Maltodextrin	12%
Honey	11%
Crisp rice	9%
Glycerine	9%
Soy Polysaccharide	7%
Oat bran	7%

C. Ensure® High Protein

Usage: ENSURE HIGH PROTEIN is a concentrated, high-protein liquid food designed for people who require additional calories, protein, vitamins, and minerals in their diets. It can be used as an oral nutritional supplement with or between meals or, in appropriate amounts, as a meal replacement. ENSURE HIGH PROTEIN is lactose- and gluten-free, and is suitable for use by people recovering from general surgery or hip fractures and by patients at risk for pressure ulcers.

Patient Conditions

For patients who require additional calories, protein, vitamins, and minerals, such as patients recovering from general surgery or hip fractures, patients at risk for pressure ulcers, and patients on low-cholesterol diets

Features

Low in saturated fat

Contains 6 g of total fat and <5 mg of cholesterol per serving

Rich, creamy taste

Excellent source of protein, calcium, and other essential vitamins and minerals

For low-cholesterol diets

Lactose-free, easily digested

Ingredients

Vanilla Supreme: -D Water, Sugar (Sucrose), Maltodextrin (Corn), Calcium and Sodium Caseinates, High-Oleic Safflower Oil, Soy Protein Isolate, Soy Oil, Canola Oil, Potassium Citrate, Calcium Phosphate Tribasic, Sodium Citrate, Magnesium Chloride, Magnesium Phosphate Dibasic, Artificial Flavor, Sodium Chloride, Soy Lecithin, Choline Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Suffate, Alpha-Tocopheryl Acetate, Gellan Gum, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Sodium Molybdate, Chromium Chloride, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein

The protein source is a blend of two high-biologic-value proteins: casein and soy.

Sodium and calcium caseinates	85%
Soy protein isolate	15%

Fat

The fat source is a blend of three oils: high-oleic safflower, canola, and soy.

High-oleic safflower oil	40%
Canola oil	30%
Soy oil	30%

The level of fat in ENSURE HIGH PROTEIN meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE HIGH PROTEIN represent 24% of the total calories, with 2.6% of the fat being from saturated fatty acids and 7.9% from polyunsaturated fatty acids. These values are within the AHA guidelines of <30% of total calories from fat, <10 of the calories from saturated fatty acids, and <10% of total calories from polyunsaturated fatty acids.

Carbohydrate

ENSURE HIGH PROTEIN contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla supreme, chocolate royal, wild berry, and

banana), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and Other Nonchocolate Flavors

Sucrose	60%
Maltodextrin	40%

Chocolate

Sucrose	70%
Maltodextrin	30%

D. Ensure® Light

Usage: ENSURE LIGHT is a low-fat liquid food designed for use as an oral nutritional supplement with or between meals. ENSURE LIGHT is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

Patient Conditions

For normal-weight or overweight patients who need extra nutrition in a supplement that contains 50% less fat and 20% fewer calories than ENSURE.

For healthy adults who don't eat right and need extra nutrition.

Features

Low in fat and saturated fat

Contains 3 g of total fat per serving and <5 mg cholesterol

Rich, creamy taste

Excellent source of calcium and other essential vitamins and minerals

For low-cholesterol diets

Lactose-free, easily digested

Ingredients

French Vanilla: -D Water, Maltodextrin (Corn), Sugar (Sucrose), Calcium Caseinate, High-Oleic Safflower Oil, Canola Oil, Magnesium Chloride, Sodium Citrate, Potassium Citrate, Potassium Phosphate Dibasic, Magnesium Phosphate Dibasic, Natural and Artificial Flavor, Calcium Phosphate Tribasic, Cellulose Gel, Choline Chloride, Soy Lecithin, Carrageenan, Salt (Sodium Chloride), Ascorbic Acid, Cellulose Gum, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium Pantothenate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Vitamin A Palmitate, Pyridoxine Hydrochloride, Riboflavin, Chromium Chloride, Folic Acid, Sodium Molybdate, Biotin, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein

The protein source is calcium caseinate.

Calcium caseinate	100%

Fat

The fat source is a blend of two oils: high-oleic safflower and canola.

High-oleic safflower oil	70%
Canola oil	30%

The level of fat in ENSURE LIGHT meets American Heart Association (AHA) guidelines. The 3 grams of fat in ENSURE LIGHT represent 13.5% of the total calories, with 1.4% of the fat being from saturated fatty acids and 2.6% from polyunsaturated fatty acids. These values are within the AHA guidelines of <30% of total calories from fat, <10% of the, calories from saturated fatty acids, and <10% of total calories from polyunsaturated fatty acids.

Carbohydrate

ENSURE LIGHT contains a combination of maltodextrin and sucrose. The chocolate flavor contains corn syrup as well. The mild sweetness and flavor variety (French vanilla, chocolate supreme, strawberry swirl), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and Other Nonchocolate Flavors

Sucrose	51%
Maltodextrin	49%

Chocolate

Sucrose	47.0%
Corn Syrup	26.5%
Maltodextrin	26.5%

Vitamins and Minerals

An 8-fl-oz serving of ENSURE LIGHT provides at least 25% of the RDIs for 24 key vitamins and minerals.

Caffeine

Chocolate flavor contains 2.1 mg caffeine/8 fl oz.

E. Ensure Plus®

Usage: ENSURE PLUS is a high-calorie, low-residue liquid food for use when extra calories and nutrients, but a normal concentration of protein, are needed. It is designed primarily as an oral nutritional supplement to be used with or between meals or, in appropriate amounts, as a meal replacement. ENSURE PLUS is lactose- and gluten-free. Although it is primarily an oral nutritional supplement, it can be fed by tube.

Patient Conditions

For patients who require extra calories and nutrients, but a normal concentration of protein, in a limited volume

For patients who need to gain or maintain healthy weight

Features

Rich, creamy taste

Good source of essential vitamins and minerals

Ingredients

Vanilla: -D Water, Corn Syrup, Maltodextrin (Corn), Corn Oil, Sodium and Calcium Caseinates, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride, Potassium Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial Flavor, Sodium Citrate, Potassium Chloride, Cho-

line Chloride, Ascorbic Acid, Carrageenan, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium Molybdate, Potassium Iodide, Sodium Selenite, Phylloquinone, Cyanocobalamin and Vitamin D3.

Protein

The protein source is a blend of two high-biologic-value proteins: casein and soy.

Sodium and calcium caseinates	84%
Soy protein isolate	16%

Fat

The fat source is corn oil.

Corn oil	100%

Carbohydrate

ENSURE PLUS contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, strawberry, coffee, buffer pecan, and eggnog), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla, Strawberry, Butter Pecan, and Coffee Flavors

Corn Syrup	39%
Maltodextrin	38%
Sucrose	23%

Chocolate and Eggnog Flavors

Corn Syrup	36%
Maltodextrin	34%
Sucrose	30%

Vitamins and Minerals

An 8-fl-oz serving of ENSURE PLUS provides at least 15% of the RDIs for 25 key Vitamins and minerals.

Caffeine

Chocolate flavor contains 3.1 mg Caffeine/8 fl oz. Coffee flavor contains a trace amount of caffeine.

F. Ensure Plus® HN

Usage: ENSURE PLUS HN is a nutritionally complete high-calorie, high-nitrogen liquid food designed for people with higher calorie and protein needs or limited volume tolerance. It may be used for oral supplementation or for total nutritional support by tube. ENSURE PLUS HN is lactose- and gluten-free.

Patient Conditions

- For patients with increased calorie and protein needs, such as following surgery or injury.
- For patients with limited volume tolerance and early satiety.

Features

- For supplemental or total nutrition
- For oral or tube feeding
- 1.5 CaVmL,
- High nitrogen
- Calorically dense

Ingredients

Vanilla: -D Water, Maltodextrin (Corn), Sodium and Calcium Caseinates, Corn Oil, Sugar (Sucrose), Soy Protein Isolate, Magnesium Chloride, Potassium Citrate, Calcium Phosphate Tribasic, Soy Lecithin, Natural and Artificial Flavor, Sodium Citrate, Choline Chloride, Ascorbic Acid, Taurine, L-Carnitine, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Carrageenan, Calcium Pantothenate, Manganese Sulfate, Cupric Sulfate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Chromium Chloride, Sodium Molybdate, Potassium Iodide, Sodium Selenite, Phylloquinone, Cyanocobalamin and Vitamin D3.

G. Ensure® Powder

Usage: ENSURE POWDER (reconstituted with water) is a low-residue liquid food designed primarily as an oral nutritional supplement to be used with or between meals. ENSURE POWDER is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

Patient Conditions

- For patients on modified diets
- For elderly patients at nutrition risk
- For patients recovering from illness/surgery
- For patients who need a low-residue diet

Features

- Convenient, easy to mix
- Low in saturated fat
- Contains 9 g of total fat and <5 mg of cholesterol per serving
- High in vitamins and minerals
- For low-cholesterol diets
- Lactose-free, easily digested

Ingredients: -D Corn Syrup, Maltodextrin (Corn), Sugar (Sucrose), Corn Oil, Sodium and Calcium Caseinates, Soy Protein Isolate, Artificial Flavor, Potassium Citrate, Magnesium Chloride, Sodium Citrate, Calcium Phosphate Tribasic, Potassium Chloride, Soy Lecithin, Ascorbic Acid, Choline Chloride, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Niacinamide, Calcium Pantothenate, Manganese Sulfate, Thiamine Chloride Hydrochloride, Cupric Sulfate, Pyridoxine Hydrochloride, Riboflavin, Vitamin A Palmitate, Folic Acid, Biotin, Sodium Molybdate, Chromium Chloride, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein

The protein source is a blend of two high-biologic-value proteins: casein and soy.

Sodium and calcium caseinates	84%
Soy protein isolate	16%

Fat

The fat source is corn oil.

Corn oil	100%

Carbohydrate

ENSURE POWDER contains a combination of corn syrup, maltodextrin, and sucrose. The mild sweetness of ENSURE POWDER, plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, helps to prevent flavor fatigue and aid in patient compliance.

Vanilla

Corn Syrup	35%
Maltodextrin	35%
Sucrose	30%

H. Ensure® Pudding

Usage: ENSURE PUDDING is a nutrient-dense supplement providing balanced nutrition in a nonliquid form to be used with or between meals. It is appropriate for consistency-modified diets (e.g., soft, pureed, or full liquid) or for people with swallowing impairments. ENSURE PUDDING is gluten-free.

Patient Conditions

For patients on consistency-modified diets (e.g., soft, pureed, or full liquid)

For patients with swallowing impairments

Features

Rich and creamy, good taste

Good source of essential vitamins and minerals

Convenient—needs no refrigeration

Gluten-free

Nutrient Profile per 5 oz: Calories 250, Protein 10.9%, Total Fat 34.9%, Carbohydrate 54.2%.

Ingredients

Vanilla: -D Nonfat Milk, Water, Sugar (Sucrose), Partially Hydrogenated Soybean Oil, Modified Food Starch, Magnesium Sulfate, Sodium Stearoyl Lactylate, Sodium Phosphate Dibasic, Artificial Flavor, Ascorbic Acid, Zinc Sulfate, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Choline Chloride, Niacinamide, Manganese Sulfate, Calcium Pantothenate, FD&C Yellow #5, Potassium Citrate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, FD&C Yellow #6, Folic Acid, Biotin, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein

The protein source is nonfat milk.

Nonfat milk	100%

Fat

The fat source is hydrogenated soybean oil.

Hydrogenated soybean oil	100%

Carbohydrate

ENSURE PUDDING contains a combination of sucrose and modified food starch. The mild sweetness and flavor variety (vanilla, chocolate, butterscotch, and tapioca) help prevent flavor fatigue. The product contains 9.2 grams of lactose per serving.

Vanilla and Other Nonchocolate Flavors

Sucrose	56%
Lactose	27%
Modified food starch	17%

Chocolate

Sucrose	58%
Lactose	26%
Modified food starch	16%

I. Ensure® with Fiber

Usage: ENSURE WITH FIBER is a fiber-containing, nutritionally complete liquid food designed for people who can benefit from increased dietary fiber and nutrients. ENSURE WITH FIBER is suitable for people who do not require a low-residue diet. It can be fed orally or by tube, and can be used as a nutritional supplement to a regular diet or, in appropriate amounts, as a meal replacement. ENSURE WITH FIBER is lactose- and gluten-free, and is suitable for use in modified diets, including low-cholesterol diets.

Patient Conditions

For patients who can benefit from increased dietary fiber and nutrients

Features

New advanced formula-low in saturated fat, higher in vitamins and minerals

Contains 6 g of total fat and <5 mg of cholesterol per serving

Rich, creamy taste

Good source of fiber

Excellent source of essential vitamins and minerals

For low-cholesterol diets

Lactose- and gluten-free

Ingredients

Vanilla: -D Water; Maltodextrin (Corn), Sugar (Sucrose), Sodium and Calcium Caseinates, Oat Fiber, High-Oleic Safflower Oil, Canola Oil, Soy Protein Isolate, Corn Oil, Soy Fiber, Calcium Phosphate Tribasic, Magnesium Chloride, Potassium Citrate, Cellulose Gel, Soy Lecithin, Potassium Phosphate Dibasic, Sodium Citrate, Natural and Artificial Flavors, Choline Chloride, Magnesium Phosphate, Ascorbic Acid, Cellulose Gum, Potassium Chloride, Carrageenan, Ferrous Sulfate, Alpha-Tocopheryl Acetate, Zinc Sulfate, Niacinamide, Manganese Sulfate, Calcium Pantothenate, Cupric Sulfate, Vitamin A Palmitate, Thiamine Chloride Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Chromium Chloride, Biotin, Sodium Molybdate, Potassium Iodide, Sodium Selenate, Phylloquinone, Vitamin D3 and Cyanocobalamin.

Protein

The protein source is a blend of two high-biologic-value proteins-casein and soy.

Sodium and calcium caseinates	80%
Soy protein isolate	20%

Fat

The fat source is a blend of three oils: high-oleic safflower, canola, and corn.

High-oleic safflower oil	40%
Canola oil	40%
Corn oil	20%

The level of fat in ENSURE WITH FIBER meets American Heart Association (AHA) guidelines. The 6 grams of fat in ENSURE WITH FIBER represent 22% of the total calories, with 2.01% of the fat being from saturated fatty acids and 6.7% from polyunsaturated fatty acids. These values are within the AHA guidelines of ≦30% of total calories from fat, <10% of the calories from saturated fatty acids, and ≦10% of total calories from polyunsaturated fatty acids.

Carbohydrate

ENSURE WITH FIBER contains a combination of maltodextrin and sucrose. The mild sweetness and flavor variety (vanilla, chocolate, and butter pecan), plus VARI-FLAVORS® Flavor Pacs in pecan, cherry, strawberry, lemon, and orange, help to prevent flavor fatigue and aid in patient compliance.

Vanilla and Other Nonchocolate Flavors

Maltodextrin	66%
Sucrose	25%
Oat Fiber	7%
Soy Fiber	2%

Chocolate

Maltodextrin	55%
Sucrose	36%
Oat Fiber	7%
Soy Fiber	2%

Fiber

The fiber blend used in ENSURE WITH FIBER consists of oat fiber and soy polysaccharide. This blend results in approximately 4 grams of total dietary fiber per 8-fl. oz can. The ratio of insoluble to soluble fiber is 95:5.

The various nutritional supplements described above and known to others of skill in the art can be substituted and/or supplemented with the PUFAs produced in accordance with the present invention.

J. Oxepa™ Nutritional Product

Oxepa is a low-carbohydrate, calorically dense, enteral nutritional product designed for the dietary management of patients with or at risk for ARDS. It has a unique combination of ingredients, including a patented oil blend containing eicosapentaenoic acid (EPA from fish oil), γ-linolenic acid (GLA from borage oil), and elevated antioxidant levels.

Caloric Distribution

Caloric density is high at 1.5 Cal/mL (355 Cal/8 fl oz), to minimize the volume required to meet energy needs. The distribution of Calories in Oxepa is shown in Table IV.

TABLE IV

Caloric Distribution of Oxepa

	per 8 fl oz.	per liter	% of Cal
Calories	355	1,500	—
Fat (g)	22.2	93.7	55.2
Carbohydrate (g)	25	105.5	28.1
Protein (g)	14.8	62.5	16.7
Water (g)	186	785	—

Fat

Oxepa contains 22.2 g of fat per 8-fl oz serving (93.7 g/L).

The fat source is an oil blend of 31.8% canola oil, 25% medium-chain triglycerides (MCTs), 20% borage oil, 20% fish oil, and 3.2% soy lecithin. The typical fatty acid profile of Oxepa is shown in Table V.

Oxepa provides a balanced amount of polyunsaturated, monounsaturated, and saturated fatty acids, as shown in Table VI.

Medium-chain trigylcerides (MCTS)—25% of the fat blend—aid gastric emptying because they are absorbed by the intestinal tract without emulsification by bile acids.

The various fatty acid components of Oxepa™ nutritional product can be substituted and/or supplemented with the PUFAs produced in accordance with this invention.

TABLE V

Typical Fatty Acid Profile

	% Total Fatty Acids	g/8 fl oz*	9/L*
Caproic (6:0)	0.2	0.04	0.18
Caprylic (8:0)	14.69	3.1	13.07
Capric (10:0)	11.06	2.33	9.87
Palmitic (16:0)	5.59	1.18	4.98
Palmitoleic	1.82	0.38	1.62
Stearic	1.94	0.39	1.64
Oleic	24.44	5.16	21.75
Linoleic	16.28	3.44	14.49
α-Linolenic	3.47	0.73	3.09
γ-Linolenic	4.82	1.02	4.29
Eicosapentaenoic	5.11	1.08	4.55
n-3-Docosapentaenoic	0.55	0.12	0.49
Docosahexaenoic	2.27	0.48	2.02
Others	7.55	1.52	6.72

Fatty acids equal approximately 95% of total fat.

TABLE VI

Fat Profile of Oxepa.

% of total calories from fat	55.2
Polyunsaturated fatty acids	31.44 g/L
Monounsaturated fatty acids	25.53 g/L
Saturated fatty acids	32.38 g/L
n-6 to n-3 ratio	1.75:1
Cholesterol	9.49 mg/8 fl oz
	40.1 mg/L

Carbohydrate

The carbohydrate content is 25.0 g per 8-fl-oz serving (105.5 g/L).

The carbohydrate sources are 45% maltodextrin (a complex carbohydrate) and 55% sucrose (a simple sugar), both of which are readily digested and absorbed.

The high-fat and low-carbohydrate content of Oxepa is designed to minimize carbon dioxide (CO2) production. High CO2 levels can complicate weaning in ventilator-dependent patients. The low level of carbohydrate also may be useful for those patients who have developed stress-induced hyperglycemia.

Oxepa is lactose-free.

Dietary carbohydrate, the amino acids from protein, and the glycerol moiety of fats can be converted to glucose within the body. Throughout this process, the carbohydrate requirements of glucose-dependent tissues (such as the central nervous system and red blood cells) are met. However, a diet free of carbohydrates can lead to ketosis, excessive catabolism of tissue protein, and loss of fluid and electrolytes. These effects can be prevented by daily ingestion of 50 to 100 g of digestible carbohydrate, if caloric intake is adequate. The carbohydrate level in Oxepa is also sufficient to minimize gluconeogenesis, if energy needs are being met.

Protein

Oxepa contains 14.8 g of protein per 8-fl-oz serving (62.5 g/L).

The total calorie/nitrogen ratio (150:1) meets the need of stressed patients.

Oxepa provides enough protein to promote anabolism and the maintenance of lean body mass without precipitating respiratory problems. High protein intakes are a concern in patients with respiratory insufficiency. Although protein has little effect on CO2 production, a high protein diet will increase ventilatory drive.

The protein sources of Oxepa are 86.8% sodium caseinate and 13.2% calcium caseinate.

The amino acid profile of the protein system in Oxepa meets or surpasses the standard for high quality protein set by the National Academy of Sciences.

Oxepa is gluten-free.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 60

<210> SEQ ID NO 1
<211> LENGTH: 1335
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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aaggtgtaca acatcagcga gttcacccgc cggcatccag ggggctcccg ggtcatcagc 180

cactacgccg ggcaggatgc cacggatccc tttgtggcct tccacatcaa caagggcctt 240

gtgaagaagt atatgaactc tctcctgatt ggagaactgt ctccagagca gcccagcttt 300

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ggccttttct tcatagtcag gttcctggaa agcaactggt ttgtgtgggt gacacagatg 1020

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tatcttcacc aataa 1335

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<211> LENGTH: 1219
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

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ttacatagta aaagacttgg actggaaatg ggtcatattt ggggcctatg cgtttggcag 180

ttgcattaac cactcaatga ctctggctat tcatgagatt gcccacaatg ctgcctttgg 240

caactgcaaa gcaatgtgga atcgctggtt tggaatgttt gctaatcttc ctattgggat 300

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aatagcagct gaatactatg acaacctccc tcactacaat tcctggataa aagtactgta 840

tgattttgtg atggatgata caataagtcc ctactcaaga atgaagaggc accaaaaagg 900

agagatggtg ctggagtaaa tatcattagt gccaaaggga ttcttctcca aaactttaga 960

tgataaaatg gaatttttgc attattaaac ttgagaccag tgatgctcag aagctcccct 1020

ggcacaattt cagagtaaga gctcggtgat accaagaagt gaatctggct tttaaacagt 1080

cagcctgact ctgtactgct cagtttcact cacaggaaac ttgtgacttg tgtattatcg 1140

tcattgagga tgtttcactc atgtctgtca ttttataagc atatcattta aaaagcttct 1200

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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gaaccatatt cccatgcaca ttgatcatga ccggaacatg gactgggttt ccacccagct 180

ccaggccaca tgcaatgtcc acaagtctgc cttcaatgac tggttcagtg gacacctcaa 240

cttccagatt gagcaccatc ttttcccac gatgcctcga cacaattacc acaaagtggc 300

tcccctggtg cagtccttgt gtgccaagca tggcatagag taccagtcca agcccctgct 360

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ctatcttcac caataacaac agccaccctg cccagtctgg aagaagagga ggaagactct 480

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gttgggtttg gggacataaa gcctctgact caaactcctc ccttttatct tctagccaca 600

gttctaagac ccaaagtggg gggtggacac agaagtccct aggagggaag gagct 655

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<211> LENGTH: 304
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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cccaagtgga accaccttgt ccacaaattc gtcattggcc acttaaaggg tgcctctgcc 180

aactggtgga atcatcgcca cttccagcac cacgccaagc ctaacatctt ccacaaggat 240

cccgatgtga acatgctgca cgtgtttgtt ctgggcgaat ggcagcccat cgagtacggc 300

aaga 304

<210> SEQ ID NO 5
<211> LENGTH: 918
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (755)...(755)
<223> OTHER INFORMATION: r = g or a at position 755

<400> SEQUENCE: 5

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gagcggtggc tagtgatcga ccgtaaggtg tacaacatca gcgagttcac ccgccggcat 120

ccaggggget cccgggtcat cagccactac gccgggcagg atgccacgga tccctttgtg 180

gccttccaca tcaacaaggg ccttgtgaag aagtatatga actctctcct gattggagaa 240

ctgtctccag agcagcccag ctttgagccc accaagaata aagagctgac agatgagttc 300

cgggagctgc gggccacagt ggagcggatg gggctcatga aggccaacca tgtcttcttc 360

ctgctgtacc tgctgcacat cttgctgctg gatggtgcag cctggctcac cctttgggtc 420

tttgggacgt ccttttgcc cttcctcctc tgtgcggtgc tgctcagtgc agttcaggcc 480

caggctggct ggctgcagca tgactttggg cacctgtcgg tcttcagcac ctcaaagtgg 540

aaccatctgc tacatcattt tgtgattggc cacctgaagg gggcccccgc cagttggtgg 600

aaccacatgc acttccagca ccatgccaag cccaactgct tccgcaaaga cccagacatc 660

aacatgcatc ccttcttctt tgccttgggg aagatcctct ctgtggagct tgggaaacag 720

aagaaaaaat atatgccgta caaccaccag cacaratact tcttcctaat tgggccccca 780

gccttgctgc ctctctactt ccagtggtat attttctatt ttgttatcca gcgaaagaag 840

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accgcaaatg cttctaaa 918

<210> SEQ ID NO 6
<211> LENGTH: 1686
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

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aatggcagcc catcgagtac ggcaagaaga agctgaaata cctgccctac aatcaccagc 180

acgaatactt cttcctgatt gggccgccgc tgctcatccc catgtatttc cagtaccaga 240

tcatcatgac catgatcgtc cataagaact gggtggacct ggcctgggcc gtcagctact 300

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tcaacttcat caggttcctg gagagccact ggtttgtgtg ggtcacacag atgaatcaca 420

tcgtcatgga gattgaccag gaggcctacc gtgactggtt cagtagccag ctgacagcca 480

cctgcaacgt ggagcagtcc ttcttcaacg actggttcag tggacacctt aacttccaga 540

ttgagcacca cctcttcccc accatgcccc ggcacaactt acacaagatc gccccgctgg 600

tgaagtctct atgtgccaag catggcattg aataccagga gaagccgcta ctgagggccc 660

tgctggacat catcaggtcc ctgaagaagt ctgggaagct gtggctggac gcctaccttc 720

acaaatgaag ccacagcccc cgggacaccg tggggaaggg gtgcaggtgg ggtgatggcc 780

agaggaatga tgggcttttg ttctgagggg tgtccgagag gctggtgtat gcactgctca 840

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gaggcagtgg ccacgttcag ggaggggccg gctggcctgg aggctcagcc caccctccag 1560

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gccctg 1686

<210> SEQ ID NO 7
<211> LENGTH: 1843
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

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cccaagtgga accaccttgt ccacaaattc gtcattggcc acttaaaggg tgcctctgcc 180

aactggtgga atcatcgcca cttccagcac cacgccaagc ctaacatctt ccacaaggat 240

cccgatgtga acatgctgca cgtgtttgtt ctgggcgaat ggcagcccat cgagtacggc 300

aagaagaagc tgaaatacct gccctacaat caccagcacg aatacttctt cctgattggg 360

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ccgccgctgc tcatccccat gtatttccag taccagatca tcatgaccat gatcgtccat 420

aagaactggg tggacctggc ctgggccgtc agctactaca tccggttctt catcacctac 480

atccctttct acggcatcct gggagccctc cttttcctca acttcatcag gttcctggag 540

agccactggt ttgtgtgggt cacacagatg aatcacatcg tcatggagat tgaccaggag 600

gcctaccgtg actggttcag tagccagctg acagccacct gcaacgtgga gcagtccttc 660

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ggcattgaat accaggagaa gccgctactg agggccctgc tggacatcat caggtccctg 840

aagaagtctg ggaagctgtg gctggacgcc taccttcaca aatgaagcca cagcccccgg 900

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<210> SEQ ID NO 8
<211> LENGTH: 2257
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

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gcccaagctg gatggctgca acatgattat ggccacctgt ctgtctacag aaaacccaag 540

tggaaccacc ttgtccacaa attcgtcatt ggccacttaa agggtgcctc tgccaactgg 600

tggaatcatc gccacttcca gcaccacgcc aagcctaaca tcttccacaa ggatcccgat 660

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<210> SEQ ID NO 9
<211> LENGTH: 432
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (432)...(432)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 432

<400> SEQUENCE: 9

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20 25 30

Ile Ser Glu Phe Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser
35 40 45

His Tyr Ala Gly Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile
50 55 60

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Asn Lys Gly Leu Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu
65                  70                  75                  80

Leu Ser Pro Glu Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu
                85                  90                  95

Thr Asp Glu Phe Arg Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu
            100                 105                 110

Met Lys Ala Asn His Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu
        115                 120                 125

Leu Leu Asp Gly Ala Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser
    130                 135                 140

Phe Leu Pro Phe Leu Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala
145                 150                 155                 160

Gln Ala Gly Trp Leu Gln His Asp Tyr Gly His Leu Ser Val Tyr Arg
                165                 170                 175

Lys Pro Lys Trp Asn His Leu Val His Lys Phe Val Ile Gly His Leu
            180                 185                 190

Lys Gly Ala Ser Ala Asn Trp Trp Asn His Arg His Phe Gln His His
        195                 200                 205

Ala Lys Pro Asn Ile Phe His Lys Asp Pro Asp Val Asn Met Leu His
    210                 215                 220

Val Phe Val Leu Gly Glu Trp Gln Pro Ile Glu Tyr Gly Lys Lys Lys
225                 230                 235                 240

Leu Lys Tyr Leu Pro Tyr Asn His Gln His Glu Tyr Phe Phe Leu Ile
                245                 250                 255

Gly Pro Pro Leu Leu Ile Pro Met Tyr Phe Gln Tyr Gln Ile Ile Met
            260                 265                 270

Thr Met Ile Val His Lys Asn Trp Val Asp Leu Ala Trp Ala Val Ser
        275                 280                 285

Tyr Tyr Ile Arg Phe Phe Ile Thr Tyr Ile Pro Phe Tyr Gly Ile Leu
    290                 295                 300

Gly Ala Leu Leu Phe Leu Asn Phe Ile Arg Phe Leu Glu Ser His Trp
305                 310                 315                 320

Phe Val Trp Val Thr Gln Met Asn His Ile Val Met Glu Ile Asp Gln
                325                 330                 335

Glu Ala Tyr Arg Asp Trp Phe Ser Ser Gln Leu Thr Ala Thr Cys Asn
            340                 345                 350

Val Glu Gln Ser Phe Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe
        355                 360                 365

Gln Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Leu His
    370                 375                 380

Lys Ile Ala Pro Leu Val Lys Ser Leu Cys Ala Lys His Gly Ile Glu
385                 390                 395                 400

Tyr Gln Glu Lys Pro Leu Leu Arg Ala Leu Leu Asp Ile Ile Arg Ser
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Leu Lys Lys Ser Gly Lys Leu Trp Leu Asp Ala Tyr Leu His Lys Xaa
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<210> SEQ ID NO 10
<211> LENGTH: 458
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (458)...(458)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 458
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<400> SEQUENCE: 10

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Leu Met Ile Ile Asp Asn Lys Val Tyr Asp Val Arg Glu Phe Val Pro
        35                  40                  45

Asp His Pro Gly Gly Ser Val Ile Leu Thr His Val Gly Lys Asp Gly
    50                  55                  60

Thr Asp Val Phe Asp Thr Phe His Pro Glu Ala Ala Trp Glu Thr Leu
65                  70                  75                  80

Ala Asn Phe Tyr Val Gly Asp Ile Asp Glu Ser Asp Arg Asp Ile Lys
                85                  90                  95

Asn Asp Asp Phe Ala Ala Glu Val Arg Lys Leu Arg Thr Leu Phe Gln
            100                 105                 110

Ser Leu Gly Tyr Tyr Asp Ser Ser Lys Ala Tyr Tyr Ala Phe Lys Val
        115                 120                 125

Ser Phe Asn Leu Cys Ile Trp Gly Leu Ser Thr Val Ile Val Ala Lys
    130                 135                 140

Trp Gly Gln Thr Ser Thr Leu Ala Asn Val Leu Ser Ala Ala Leu Leu
145                 150                 155                 160

Gly Leu Phe Trp Gln Gln Cys Gly Trp Leu Ala His Asp Phe Leu His
                165                 170                 175

His Gln Val Phe Gln Asp Arg Phe Trp Gly Asp Leu Phe Gly Ala Phe
            180                 185                 190

Leu Gly Gly Val Cys Gln Gly Phe Ser Ser Ser Trp Trp Lys Asp Lys
        195                 200                 205

His Asn Thr His His Ala Ala Pro Asn Val His Gly Glu Asp Pro Asp
    210                 215                 220

Ile Asp Thr His Pro Leu Leu Thr Trp Ser Glu His Ala Leu Glu Met
225                 230                 235                 240

Phe Ser Asp Val Pro Asp Glu Glu Leu Thr Arg Met Trp Ser Arg Phe
                245                 250                 255

Met Val Leu Asn Gln Thr Trp Phe Tyr Phe Pro Ile Leu Ser Phe Ala
            260                 265                 270

Arg Leu Ser Trp Cys Leu Gln Ser Ile Leu Phe Val Leu Pro Asn Gly
        275                 280                 285

Gln Ala His Lys Pro Ser Gly Ala Arg Val Pro Ile Ser Leu Val Glu
    290                 295                 300

Gln Leu Ser Leu Ala Met His Trp Thr Trp Tyr Leu Ala Thr Met Phe
305                 310                 315                 320

Leu Phe Ile Lys Asp Pro Val Asn Met Leu Val Tyr Phe Leu Val Ser
                325                 330                 335

Gln Ala Val Cys Gly Asn Leu Leu Ala Ile Val Phe Ser Leu Asn His
            340                 345                 350

Asn Gly Met Pro Val Ile Ser Lys Glu Glu Ala Val Asp Met Asp Phe
        355                 360                 365

Phe Thr Lys Gln Ile Ile Thr Gly Arg Asp Val His Pro Gly Leu Phe
    370                 375                 380

Ala Asn Trp Phe Thr Gly Gly Leu Asn Tyr Gln Ile Glu His His Leu
385                 390                 395                 400

Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro Ala Val
                405                 410                 415
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Glu Thr Leu Cys Lys Lys Tyr Asn Val Arg Tyr His Thr Thr Gly Met
            420                 425                 430

Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val Ser Lys
        435                 440                 445

Ala Ala Ser Lys Met Gly Lys Ala Gln Xaa
    450                 455


<210> SEQ ID NO 11
<211> LENGTH: 444
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Met Ala Pro Asp Pro Val Ala Ala Glu Thr Ala Ala Gln Gly Pro Thr
 1               5                  10                  15

Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln Arg Ser Gly Cys Glu
            20                  25                  30

Glu Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn Ile Ser Glu Phe
        35                  40                  45

Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser His Tyr Ala Gly
    50                  55                  60

Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile Asn Lys Gly Leu
65                  70                  75                  80

Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu Leu Ser Pro Glu
                85                  90                  95

Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe
            100                 105                 110

Arg Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn
        115                 120                 125

His Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly
    130                 135                 140

Ala Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe
145                 150                 155                 160

Leu Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala Gln Ala Gly Trp
                165                 170                 175

Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr Ser Lys Trp
            180                 185                 190

Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys Gly Ala Pro
        195                 200                 205

Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala Lys Pro Asn
    210                 215                 220

Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe Phe Phe Ala
225                 230                 235                 240

Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys Lys Lys Tyr
                245                 250                 255

Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile Gly Pro Pro
            260                 265                 270

Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr Phe Val Ile
        275                 280                 285

Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr Phe Tyr Val
    290                 295                 300

Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys Ala Phe Leu
305                 310                 315                 320

Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp Phe Val Trp
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325 330 335

Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His Asp Arg Asn
340 345 350

Met Asp Trp Val Ser Thr Gln Leu Leu Ala Thr Cys Asn Val His Lys
355 360 365

Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
370 375 380

His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His Lys Val Ala
385 390 395 400

Pro Leu Val Gln Ser Leu Cys Ala Lys Arg Gly Ile Glu Tyr Gln Ser
405 410 415

Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser Leu Lys Glu
420 425 430

Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln
435 440

<210> SEQ ID NO 12
<211> LENGTH: 864
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

ctcctggagc ccgtcagtat cggcggaatt ccggcagttc aggcccaggc tggctggctg 60

cagcatgact ttgggcacct gtcggtcttc agcacctcaa agtggaacca tctgctacat 120

cattttgtga ttggccacct gaaggggcc cccgccagtt ggtggaacca catgcacttc 180

cagcaccatg ccaagcccaa ctgcttccgc aaagacccag acatcaacat gcatcccttc 240

ttctttgcct tggggaagat cctctctgtg gagcttggga aacagaagaa aaaatatatg 300

ccgtacaacc accagcacaa atacttcttc ctaattgggc ccccagcctt gctgcctctc 360

tacttccagt ggtatatttt ctattttgtt atccagcgaa agaagtgggt ggacttggcc 420

tggatgatta ccttctacgt ccgcttcttc ctcacttatg tgccactatt ggggctgaaa 480

gccttcctgg gccttttctt catagtcagg ttcctggaaa gcaactggtt tgtgtgggtg 540

acacagatga accatattcc catgcacatt gatcatgacc ggaacatgga ctgggtttcc 600

acccagctcc aggccacatg caatgtccac aagtctgcct tcaatgactg gttcagtgga 660

cacctcaact tccagattga gcaccatctt tttcccacga tgcctcgaca caattaccac 720

aaagtggctc ccctggtgca gtccttgtgt gccaagcatg gcatagagta ccagtccaag 780

cccctgctgt cagccttcgc cgacatcatc cactcactaa aggagtcagg gcagctctgg 840

ctagatgcct atcttcacca ataa 864

<210> SEQ ID NO 13
<211> LENGTH: 287
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

Leu Leu Glu Pro Val Ser Ile Gly Gly Ile Pro Ala Val Gln Ala Gln
1 5 10 15

Ala Gly Trp Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr
20 25 30

Ser Lys Trp Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys
35 40 45

Gly Ala Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala

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    50                  55                  60

Lys Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe
65                  70                  75                  80

Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys
                85                  90                  95

Lys Lys Tyr Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile
            100                 105                 110

Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr
        115                 120                 125

Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr
    130                 135                 140

Phe Tyr Val Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys
145                 150                 155                 160

Ala Phe Leu Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp
                165                 170                 175

Phe Val Trp Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His
            180                 185                 190

Asp Arg Asn Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn
        195                 200                 205

Val His Lys Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe
    210                 215                 220

Gln Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His
225                 230                 235                 240

Lys Val Ala Pro Leu Val Gln Ser Leu Cys Ala Lys His Gly Ile Glu
                245                 250                 255

Tyr Gln Ser Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser
            260                 265                 270

Leu Lys Glu Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln
        275                 280                 285


<210> SEQ ID NO 14
<211> LENGTH: 288
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (288)...(288)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 288

<400> SEQUENCE: 14

Leu Leu Glu Pro Val Ser Ile Gly Gly Ile Pro Ala Val Gln Ala Gln
 1               5                  10                  15

Ala Gly Trp Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr
            20                  25                  30

Ser Lys Trp Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys
        35                  40                  45

Gly Ala Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala
    50                  55                  60

Lys Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe
65                  70                  75                  80

Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys
                85                  90                  95

Lys Lys Tyr Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile
            100                 105                 110

Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr
        115                 120                 125
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Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr
    130                 135                 140

Phe Tyr Val Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys
145                 150                 155                 160

Ala Phe Leu Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp
                165                 170                 175

Phe Val Trp Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His
            180                 185                 190

Asp Arg Asn Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn
        195                 200                 205

Val His Lys Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe
    210                 215                 220

Gln Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His
225                 230                 235                 240

Lys Val Ala Pro Leu Val Gln Ser Leu Cys Ala Lys His Gly Ile Glu
                245                 250                 255

Tyr Gln Ser Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser
            260                 265                 270

Leu Lys Glu Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln Xaa
        275                 280                 285


<210> SEQ ID NO 15
<211> LENGTH: 315
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (315)...(315)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 315

<400> SEQUENCE: 15

Ala Ser Tyr Tyr Ala Gln Leu Phe Val Pro Phe Val Val Glu Arg Thr
 1               5                  10                  15

Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe Ala Cys Ala Gln
            20                  25                  30

Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe Ser Val Thr His
        35                  40                  45

Asn Pro Thr Val Trp Lys Ile Leu Gly Ala Thr His Asp Phe Phe Asn
    50                  55                  60

Gly Ala Ser Tyr Leu Val Trp Met Tyr Gln His Met Leu Gly His His
65                  70                  75                  80

Pro Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val Ser Thr Ser Glu
                85                  90                  95

Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp Phe Val Asn His
            100                 105                 110

Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly Leu Leu Ala Phe
        115                 120                 125

Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe Val Lys Thr Asn
    130                 135                 140

Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His Thr Val Met Phe
145                 150                 155                 160

Trp Gly Gly Lys Ala Phe Phe Val Trp Tyr Arg Leu Ile Val Pro Leu
                165                 170                 175

Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe Thr Val Ala Asp
            180                 185                 190
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Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln Ala Asn His Val
        195                 200                 205

Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn Gly Ile Ile Gln
    210                 215                 220

Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln Asp Tyr Ala His
225                 230                 235                 240

Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu Asn Tyr Gln Ala
                245                 250                 255

Val His His Leu Phe Pro Asn Val Ser Gln His His Tyr Pro Asp Ile
            260                 265                 270

Leu Ala Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys Val Pro Tyr Leu
        275                 280                 285

Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His Leu Glu His Leu
    290                 295                 300

Arg Val Leu Gly Leu Arg Pro Lys Glu Glu Xaa
305                 310                 315


<210> SEQ ID NO 16
<211> LENGTH: 288
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (288)...(288)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 288

<400> SEQUENCE: 16

Leu Leu Glu Pro Val Ser Ile Gly Gly Ile Pro Ala Val Gln Ala Gln
 1               5                  10                  15

Ala Gly Trp Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr
            20                  25                  30

Ser Lys Trp Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys
        35                  40                  45

Gly Ala Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala
    50                  55                  60

Lys Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe
65                  70                  75                  80

Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys
                85                  90                  95

Lys Lys Tyr Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile
            100                 105                 110

Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr
        115                 120                 125

Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr
    130                 135                 140

Phe Tyr Val Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys
145                 150                 155                 160

Ala Phe Leu Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp
                165                 170                 175

Phe Val Trp Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His
            180                 185                 190

Asp Arg Asn Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn
        195                 200                 205

Val His Lys Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe
    210                 215                 220

Gln Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His
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225                 230                 235                 240

Lys Val Ala Pro Leu Val Gln Ser Leu Cys Ala Lys His Gly Ile Glu
                245                 250                 255

Tyr Gln Ser Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser
            260                 265                 270

Leu Lys Glu Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln Xaa
        275                 280                 285


<210> SEQ ID NO 17
<211> LENGTH: 323
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (323)...(323)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 323

<400> SEQUENCE: 17

Gly Leu Ser Thr Val Ile Val Ala Lys Trp Gly Gln Thr Ser Thr Leu
 1               5                  10                  15

Ala Asn Val Leu Ser Ala Ala Leu Leu Gly Leu Phe Trp Gln Gln Cys
            20                  25                  30

Gly Trp Leu Ala His Asp Phe Leu His His Gln Val Phe Gln Asp Arg
        35                  40                  45

Phe Trp Gly Asp Leu Phe Gly Ala Phe Leu Gly Gly Val Cys Gln Gly
    50                  55                  60

Phe Ser Ser Ser Trp Trp Lys Asp Lys His Asn Thr His His Ala Ala
65                  70                  75                  80

Pro Asn Val His Gly Glu Asp Pro Asp Ile Asp Thr His Pro Leu Leu
                85                  90                  95

Thr Trp Ser Glu His Ala Leu Glu Met Phe Ser Asp Val Pro Asp Glu
            100                 105                 110

Glu Leu Thr Arg Met Trp Ser Arg Phe Met Val Leu Asn Gln Thr Trp
        115                 120                 125

Phe Tyr Phe Pro Ile Leu Ser Phe Ala Arg Leu Ser Trp Cys Leu Gln
    130                 135                 140

Ser Ile Leu Phe Val Leu Pro Asn Gly Gln Ala His Lys Pro Ser Gly
145                 150                 155                 160

Ala Arg Val Pro Ile Ser Leu Val Glu Gln Leu Ser Leu Ala Met His
                165                 170                 175

Trp Thr Trp Tyr Leu Ala Thr Met Phe Leu Phe Ile Lys Asp Pro Val
            180                 185                 190

Asn Met Leu Val Tyr Phe Leu Val Ser Gln Ala Val Cys Gly Asn Leu
        195                 200                 205

Leu Ala Ile Val Phe Ser Leu Asn His Asn Gly Met Pro Val Ile Ser
    210                 215                 220

Lys Glu Glu Ala Val Asp Met Asp Phe Phe Thr Lys Gln Ile Ile Thr
225                 230                 235                 240

Gly Arg Asp Val His Pro Gly Leu Phe Ala Asn Trp Phe Thr Gly Gly
                245                 250                 255

Leu Asn Tyr Gln Ile Glu His His Leu Phe Pro Ser Met Pro Arg His
            260                 265                 270

Asn Phe Ser Lys Ile Gln Pro Ala Val Glu Thr Leu Cys Lys Lys Tyr
        275                 280                 285

Asn Val Arg Tyr His Thr Thr Gly Met Ile Glu Gly Thr Ala Glu Val
    290                 295                 300
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Phe Ser Arg Leu Asn Glu Val Ser Lys Ala Ala Ser Lys Met Gly Lys
305                 310                 315                 320

Ala Gln Xaa


<210> SEQ ID NO 18
<211> LENGTH: 288
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (288)...(288)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 288

<400> SEQUENCE: 18

Leu Leu Glu Pro Val Ser Ile Gly Gly Ile Pro Ala Val Gln Ala Gln
 1               5                  10                  15

Ala Gly Trp Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr
            20                  25                  30

Ser Lys Trp Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys
        35                  40                  45

Gly Ala Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala
    50                  55                  60

Lys Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe
65                  70                  75                  80

Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys
                85                  90                  95

Lys Lys Tyr Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile
            100                 105                 110

Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr
        115                 120                 125

Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr
    130                 135                 140

Phe Tyr Val Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys
145                 150                 155                 160

Ala Phe Leu Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp
                165                 170                 175

Phe Val Trp Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His
            180                 185                 190

Asp Arg Asn Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn
        195                 200                 205

Val His Lys Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe
    210                 215                 220

Gln Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His
225                 230                 235                 240

Lys Val Ala Pro Leu Val Gln Ser Leu Cys Ala Lys His Gly Ile Glu
                245                 250                 255

Tyr Gln Ser Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser
            260                 265                 270

Leu Lys Glu Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln Xaa
        275                 280                 285


<210> SEQ ID NO 19
<211> LENGTH: 356
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
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<222> LOCATION: (294)...(294)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 294
<221> NAME/KEY: VARIANT
<222> LOCATION: (320)...(320)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 320

<400> SEQUENCE: 19

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Val Phe Tyr Phe Gly Asn Gly Trp Ile Pro Thr Leu Ile Thr Ala Phe
 1               5                  10                  15

Val Leu Ala Thr Ser Gln Ala Gln Ala Gly Trp Leu Gln His Asp Tyr
            20                  25                  30

Gly His Leu Ser Val Tyr Arg Lys Pro Lys Trp Asn His Leu Val His
        35                  40                  45

Lys Phe Val Ile Gly His Leu Lys Gly Ala Ser Ala Asn Trp Trp Asn
    50                  55                  60

His Arg His Phe Gln His His Ala Lys Pro Asn Ile Phe His Lys Asp
65                  70                  75                  80

Pro Asp Val Asn Met Leu His Val Phe Val Leu Gly Glu Trp Gln Pro
                85                  90                  95

Ile Glu Tyr Gly Lys Lys Lys Leu Lys Tyr Leu Pro Tyr Asn His Gln
            100                 105                 110

His Glu Tyr Phe Phe Leu Ile Gly Pro Pro Leu Leu Ile Pro Met Tyr
        115                 120                 125

Phe Gln Tyr Gln Ile Ile Met Thr Met Ile Val His Lys Asn Trp Val
    130                 135                 140

Asp Leu Ala Trp Ala Val Ser Tyr Tyr Ile Arg Phe Phe Ile Thr Ile
145                 150                 155                 160

Pro Phe Tyr Gly Ile Leu Gly Ala Leu Leu Phe Leu Asn Phe Ile Arg
                165                 170                 175

Phe Leu Glu Ser His Trp Phe Val Trp Val Thr Gln Met Asn His Ile
            180                 185                 190

Val Met Glu Ile Asp Gln Glu Ala Tyr Arg Asp Trp Phe Ser Ser Gln
        195                 200                 205

Leu Thr Ala Thr Cys Asn Val Glu Gln Ser Phe Phe Asn Asp Trp Phe
    210                 215                 220

Ser Gly His Leu Asn Phe Gln Ile Glu His His Leu Phe Pro Thr Met
225                 230                 235                 240

Pro Arg His Asn Leu His Lys Ile Ala Pro Leu Val Lys Ser Leu Cys
                245                 250                 255

Ala Lys His Gly Ile Glu Tyr Gln Glu Lys Pro Leu Leu Arg Ala Leu
            260                 265                 270

Leu Asp Ile Ile Arg Ser Leu Lys Lys Ser Gly Lys Leu Trp Leu Asp
        275                 280                 285

Ala Tyr Leu His Lys Xaa Ser His Ser Pro Arg Asp Thr Val Gly Lys
    290                 295                 300

Gly Cys Arg Trp Gly Asp Gly Gln Arg Asn Asp Gly Leu Leu Phe Xaa
305                 310                 315                 320

Gly Val Ser Glu Arg Leu Val Tyr Ala Leu Leu Thr Asp Pro Met Leu
                325                 330                 335

Asp Leu Ser Pro Phe Leu Leu Ser Phe Phe Ser Ser His Leu Pro His
            340                 345                 350

Ser Thr Leu Pro
        355
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<210> SEQ ID NO 20

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<211> LENGTH: 219
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 20

Leu Leu Glu Pro Val Ser Ile Gly Gly Ile Pro Ala Val Gln Ala Gln
1 5 10 15

Ala Gly Trp Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr
20 25 30

Ser Lys Trp Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys
35 40 45

Gly Ala Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala
50 55 60

Lys Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe
65 70 75 80

Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys
85 90 95

Lys Lys Tyr Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile
100 105 110

Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr
115 120 125

Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr
130 135 140

Phe Tyr Val Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys
145 150 155 160

Ala Phe Leu Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp
165 170 175

Phe Val Trp Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His
180 185 190

Asp Arg Asn Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn
195 200 205

Val His Lys Ser Ala Phe Asn Asp Trp Phe Ser
210 215

<210> SEQ ID NO 21
<211> LENGTH: 182
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (128)...(128)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 128

<400> SEQUENCE: 21

Leu His Ile Leu Leu Leu Asp Gly Ala Ala Trp Leu Thr Leu Trp Val
1 5 10 15

Phe Gly Thr Ser Phe Leu Pro Phe Leu Leu Cys Ala Val Leu Leu Ser
20 25 30

Ala Val Gln Ala Gln Ala Gly Trp Leu Gln His Asp Phe Gly His Leu
35 40 45

Ser Val Phe Ser Thr Ser Lys Trp Asn His Leu Leu His His Phe Val
50 55 60

Ile Gly His Leu Lys Gly Ala Pro Ala Ser Trp Trp Asn His Met His
65 70 75 80

Phe Gln His His Ala Lys Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile
85 90 95

Asn Met His Pro Phe Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu

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          100                 105                 110

Leu Gly Lys Gln Lys Lys Lys Tyr Met Pro Tyr Asn His Gln His Xaa
        115                 120                 125

Tyr Phe Phe Leu Ile Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln
    130                 135                 140

Trp Tyr Ile Phe Tyr Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu
145                 150                 155                 160

Ala Trp Ile Ser Lys Gln Glu Tyr Asp Glu Ala Gly Leu Pro Leu Ser
                165                 170                 175

Thr Ala Asn Ala Ser Lys
            180


<210> SEQ ID NO 22
<211> LENGTH: 179
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (1)...(1)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 1
<221> NAME/KEY: VARIANT
<222> LOCATION: (11)...(11)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 11
<221> NAME/KEY: VARIANT
<222> LOCATION: (19)...(19)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 19
<221> NAME/KEY: VARIANT
<222> LOCATION: (139)...(139)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 139
<221> NAME/KEY: VARIANT
<222> LOCATION: (163)...(163)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 163
<221> NAME/KEY: VARIANT
<222> LOCATION: (172)...(172)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 172

<400> SEQUENCE: 22

Xaa Leu Asp Leu Pro Thr Asn Met Met Glu Xaa Arg Lys Ala Ala Ala
 1               5                  10                  15

Glu Leu Xaa Ala Ala Glu Thr Ala Ala Gln Gly Pro Thr Pro Arg Tyr
            20                  25                  30

Phe Thr Trp Asp Glu Val Ala Gln Arg Ser Gly Cys Glu Glu Arg Trp
        35                  40                  45

Leu Val Ile Asp Arg Lys Val Tyr Asn Ile Ser Glu Phe Thr Arg Arg
    50                  55                  60

His Pro Gly Gly Ser Arg Val Ile Ser His Tyr Ala Gly Gln Asp Ala
65                  70                  75                  80

Thr Asp Pro Phe Val Ala Phe His Ile Asn Lys Gly Leu Val Lys Lys
                85                  90                  95

Tyr Met Asn Ser Leu Leu Ile Gly Glu Leu Ser Pro Glu Gln Pro Ser
            100                 105                 110

Phe Glu Pro Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe Arg Glu Leu
        115                 120                 125

Arg Ala Thr Val Glu Gln Arg Phe Pro Val Xaa Phe Leu Thr Cys Thr
    130                 135                 140

Gly Ala His Gly Phe Phe Ser Leu Glu Val Pro Gly Leu Pro Asp Ser
145                 150                 155                 160

Asn Lys Xaa Phe Ser Trp Thr Ser Arg Pro Ile Xaa Trp Asn Lys Gly
                165                 170                 175

Lys Arg Pro
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<210> SEQ ID NO 23
<211> LENGTH: 133
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 23

Ala Glu Gln Ser Asp Glu Ala Val Lys Tyr Tyr Thr Leu Glu Glu Ile
1 5 10 15

Gln Lys His Asn His Ser Lys Ser Thr Trp Leu Ile Leu His His Lys
20 25 30

Val Tyr Asp Leu Thr Lys Phe Leu Glu Glu His Pro Gly Gly Glu Glu
35 40 45

Val Leu Arg Glu Gln Ala Gly Gly Asp Ala Thr Glu Asn Phe Glu Asp
50 55 60

Val Gly His Ser Thr Asp Ala Arg Glu Met Ser Lys Thr Phe Ile Ile
65 70 75 80

Gly Glu Leu His Pro Asp Asp Arg Pro Lys Leu Asn Lys Pro Pro Glu
85 90 95

Thr Leu Ile Thr Thr Ile Asp Ser Ser Ser Ser Trp Trp Thr Asn Trp
100 105 110

Val Ile Pro Ala Ile Ser Ala Val Ala Val Ala Leu Met Tyr Arg Leu
115 120 125

Tyr Met Ala Glu Asp
130

<210> SEQ ID NO 24
<211> LENGTH: 300
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 24

cccgaccaat atgatggaat aaaggaaagc ggccgctgaa ttataggccg ccgagaccgc 60

ggctcaggga cctaccccgc gttacttcac atgggacgag gtggcccagc gctcagggtg 120

cgaggagcgg tggcttgtga tcgaccgtaa ggtgtacaac atcagcgagt tcacccgccg 180

gcatccaggg ggctcccggg tcatcagcca ctacgccggg caggatgcca cggatccctt 240

cgtggccttc cacatcaaca agggccttgt gaagaagtat atgaactctc tcctgattgg 300

<210> SEQ ID NO 25
<211> LENGTH: 300
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

cccggcgcgc ggcgtcgcca ggccagctat ggcccccgac ccggtggccg ccgagaccgc 60

ggctcaggga cctaccccgc gctacttcac ctgggacgag gtggcccagc gctcagggtg 120

cgaggagcgg tggctagtga tcgaccgtaa ggtgtacaac atcagcgagt tcacccgccg 180

gcatccaggg ggctcccggg tcatcagcca ctacgccggg caggatgcca cggtgagcgc 240

agccaggcgg gggcacagga gagggcggga ccggaggctg agtgcagggg agacagagtt 300

<210> SEQ ID NO 26
<211> LENGTH: 360
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 26

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aatacgactc actatagggc tcgagcggcc gcccgggcag gtccggacct gccaacgtga 60

atcttatcgc catggacctt accttgcaca acccaaagta gctgccttgg ggcagggggt 120

ggccagagtg cttagggaaa tgtggagccc tacccagaac aacggtggag ggaaagggaa 180

gaaacgcaga agtgccccag ttcggacgta gggaagtctt cctcttcgtg gtttttggag 240

aaccctagct aagagaggaa agggacttat tgaaagaccc gcaagaaggg acggaagtct 300

catagccctg agaggatccc tttgtggcct tccacatcaa caagggcctt gtgaagaagt 360

<210> SEQ ID NO 27
<211> LENGTH: 419
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 27

ccccgcccca cacgccgcat cacttacagg gcccggggct gccggacctg ccaacgtgaa 60

tcttatcgcc atggacctta ccttgcacaa cccaaagtag ctgccttggg gcaggggtg 120

gccagagtgc ttagggaaat gtggagccct acccagaaca acggtggagg gaaagggaag 180

aaacgcagaa gtgccccagt tcggacgtag ggaagtcttc ctcttcgtgg tttttggaga 240

accctagcta agagaggaaa gggacttatt gaaagacccg caagaaggga cggaagtcta 300

accctagcta agagaggaaa gggacttatt gaaagacccg caagaaggga cggaagtctc 360

atagccctga gaggtgaagc cagctggagt tgatgggtcg aatggggacc tagagaact 419

<210> SEQ ID NO 28
<211> LENGTH: 240
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 28

tatagggctc gagcggccgc ccgggcaggt gcccggaggc gcctgatcat acctgttgcc 60

cggtgattgg gtgtcctgcg gatgcgggat gaaaaggcgg gagagaggcc tggaaaagtg 120

gagtctgggg agtggggatg gaggccaaca acacgcacac acaaacaaag ggtcccgcct 180

ccctgccgtg cattccatct gcagccccga gcctcaggat ccctttgtgg ccttccacat 240

<210> SEQ ID NO 29
<211> LENGTH: 239
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 29

cgagccaaac accgactaat tcggaggaaa gcccggaggc gcctgatcat acctgttgcc 60

cggtgattgg gtgtcctgcg gatgcgggat gaaaaggcgg gagagaggcc tggagaagtg 120

gagtctgggg agtggggatg gaggccaaca acacgcacac acaaacaaag ggtcccgcct 180

ccctgccgtg cattccatct gcagccccga gcctcaggtc tctgggcggg gacagaacc 239

<210> SEQ ID NO 30
<211> LENGTH: 300
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 30

cgagcggccg cccgggcagg tctagaattc agcggccgct gaagccgcgt ctggacctag 60

gtgccggtct ccactcgcca gcaggagcgg agagggagca ggaaaggagc ccattctcga 120

ggatggggct gaaacgggaa gcttggggag accgctgcct tggggacccc tgcgtcgtgt 180

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gaagactgga ggacgcggaa gggacagcgc tggccgggga gggcaagcgg ccgctggcga 240

tccctttgtg gccttccaca tcaacaaggg ccttgtgaag aagtatatga actctctcct 300

<210> SEQ ID NO 31
<211> LENGTH: 299
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 31

agggagtcac atcctgtctc gatggctagg agaggcagcg cagccgcgtc tggacctagg 60

tgccggtctc cactcgccag caggagcgga gagggagcag gaaaggagcc cattctcgag 120

gatggggctg aaacgggaag cttggggaga ccgctgcctt ggggacccct gcgtcgtgtg 180

aagactggag gacgcggaag ggacagcgct ggccggggag ggcaagcggc cgctggcgta 240

cataagggat tgggaatggc atacacttag cgaggacccc cagagctgtt ctcgaatcg 299

<210> SEQ ID NO 32
<211> LENGTH: 286
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 32

ttattccctt atttgtccct gcccatgtcc tgctgattgg tccattttac ctctagctag 60

ctaaagagca cggattggtg cattttgcaa acctctggct acagaggggt tctccaggtc 120

tgcactcgac ccaggaagtc catctggctt cacctctcac ttcaacttgg gtacagcctt 180

ctggcgggca ggaagatggc ctttggtgcg aacactgccg gagtccaggg ggctggctcc 240

ctcacctttc atcttctccc ggcacttgca ggatcccttt gtggcc 286

<210> SEQ ID NO 33
<211> LENGTH: 286
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 33

atagagcact gattggtcca ttttacaggg tgctgattgg tccattttac ctctagctag 60

ctaaagagca cggattggtg cattttacaa acctctagct acagaaaagt tctccaagtc 120

tgcactcgac ccaggaagtc catctggctt cacctctcac ttcaacttgg gtacagcctt 180

ctggcgggca ggaggatggc ctttggtgcg aacactgccg gagtccaggg ggctggctcc 240

ctcacctttc atcttctccc ggcacttgca ggatcccttt gtggcc 286

<210> SEQ ID NO 34
<211> LENGTH: 4698
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 34

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acgtcaggaa gtcgaatccg gcggcgacgc ttttagggag cccgcgaggg ggcgcgtgtt 120

ggcagcccag ctgtgagttg cccaagaccc accgggggac gggatctcgc tccccgcgcc 180

acgaggctcg gccaatggga acgcgcgctg cgaggcccgc cggtctgccc tgcggtgctg 240

aaaacccggc gcgcaggcgg ctggctctgg gcgcgcgcca gcaaatccac tcctggagcc 300

cgcggacccc gagcacgcgc ctgacagccc ctgctggccc ggcgcgcggc gtcgccaggc 360

-continued

cagctatggc ccccgacccg gtggccgccg agaccgcggc tcagggacct accccgcgct 420

acttcacctg ggacgaggtg gcccagcgct cagggtgcga ggagcggtgg ctagtgatcg 480

accgtaaggt gtacaacatc agcgagttca cccgccggca tccaggggge tcccgggtca 540

tcagccacta cgccgggcag gatgccacgg tgagcgcagc caggcggggg cacaggagag 600

ggcgggaccg gaggctgagt gcaggggaga cagagttacg cactccgagc caaacaccga 660

ctaattcgga ggaaagcccg gaggcgcctg atcatacctg ttgcccggtg attgggtgtc 720

ctgcggatgc gggatgaaaa ggcgggagag aggcctggag aagtggagtc tggggagtgg 780

ggatggaggc caacaacacg cacacacaaa caaagggtcc cgcctccctg ccgtgcattc 840

catctgcagc cccgagcctc aggtctctgg gcggggacag aaccccgagc tgggtaggct 900

aggagggagg agagcaagga tgcaggccgc ctggggaggg agggggtcag tggccagggg 960

agggagtcac atcctgtctc gatggctagg agaggcagcg cagccgcgtc tggacctagg 1020

tgccggtctc cactcgccag caggagcgga gagggagcag gaaaggagcc cattctcgag 1080

gatggggctg aaacgggaag cttggggaga ccgctgcctt ggggacccct gcgtcgtgtg 1140

aagactggag gacgcggaag ggacagcgct ggccggggag ggcaagcggc cgctggcgta 1200

cataagggat tgggaatggc atacacttag cgaggacccc cagagctgtt ctcgaatcgc 1260

ggggaggccc tgagccgcag gccagcgagg tcttcagcta ttccgcggag cggaccgctg 1320

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ccaagggcgc aggcgtgtcc cggcgcatgc gcagacgaaa caggcaccaa cgctggagct 1860

tcccgcagtg tgatttgggg ccggggatgc cgcggcgggg acggcgattg gtccgtatgt 1920

gtggtgccac cggccgccgc tccgccccgg cccccgcccc acacgccgca tcacttacag 1980

ggcccggggc tgccggacct gccaacgtga atcttatcgc catggacctt accttgcaca 2040

acccaaagta gctgccttgg ggcagggggt ggccagagtg cttagggaaa tgtggagccc 2100

tacccagaac aacggtggag ggaaagggaa gaaacgcaga agtgccccag ttcggacgta 2160

gggaagtctt cctcttcgtg gtttttggag aaccctagct aagagaggaa agggacttat 2220

tgaaagaccc gcaagaaggg acggaagtct catagccctg agaggtgaag ccagctggag 2280

ttgatgggtc gaatggggac ctagagaact tttctgtatc tagaggtttg taaaatgcac 2340

caatcagtgc tctgtaaaaa cgcaccaatt ggcgctctgt agctagctag aggtttgtaa 2400

aatgagccaa tcagcaggac gtgggcaggg acaactaaga caataaaagc tggccacccc 2460

agccagctgc tgcaacccgc tccagttccc ttacaggctg tggaagcatt gttcttttgc 2520

tcgtcacact aaaccttgct gctgctcatt ctttgggtct gcaaagagtg ttattccttt 2580

aagagctata acagcgggaa ggtccacggc tccattcttg aagtcagtga gaccataccc 2640

gccggaagga accaacgccc gacacagccc cacccatctc tcctgtttct cacctatact 2700

gaaattcttg ggcaaaagct gtctgtggac acacccaggg gaaaggccag cccaggcagg 2760

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tgtttcttag tggttcccct cagccaatgc ttcccattcc ttgatgcatc cttctaacta 2820

gagcagatgc tcggtgatct taaactgtgg acacctggga gcaccctcaa aaggcagctg 2880

ggcctaggga gatggcctgt gcttctgtgt caggagttgg ttccttcagg tgggcttgtg 2940

gtctcgctga cgtcaagaat gaagccatga accttcgcgg tgagtgttac agctcttaca 3000

ggtggcgtgg acccaaagag tgagcagcag caagatttat tgtgaagagc aaagaacaaa 3060

gcttccacag cgtggaaggg tacccgagca ggttgccgct gctggacgtt ggggggtgtg 3120

agggggagca gccttttttt ttcttttttt tttgagacgg agtctccctg tcgcccaggc 3180

tggagtgcag tggcgcgatc tcggctcact gcaggctccg ccccccccc ggggttcacg 3240

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gctaattttt tgtatttta gtagagacgg ggtttcactg tgttagccag gatggtctcg 3360

atctcctgac ctcgtgatcc acccgccttg gcctcccaaa gtgctgggat tacaggcgtg 3420

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ccttccacat caacaagggc cttgtgaaga agtatatgaa ctctctcctg attggagaac 3840

tgtctccaga gcagcccagc tttgagccca ccaagaatgt aagaccctgt gtttgctatg 3900

tcgcaactat tggttgttga gggggacaga gagggggtgg aaggagagtc tagatggaat 3960

cacagtcata gtaatcacag tcagtagtag ctctggggag tcttgaggtc cctgcttctc 4020

ttgcatagtc atgaggtcac aggcccaagg gagcatggct ttgcaaccta tggctccccc 4080

aaggctgcca ctaccatggc tgccatcatt gttatcatca ttgttatcat atgagcactt 4140

actatgcacc aagcataaac tcataactct tacacattta cagatgagat aacaggctca 4200

gggaggttaa gcaacacagc caaggatcac acagttagta aatggcagag caaggactta 4260

gtcccctgaa ctcttaggca ctatcccatg gcacctcctc ctgtcatcct cattgtcgtg 4320

gtatctttgc ctaggactgt ggacttccca cagctacctc agtgggaggt ccttgagcct 4380

gagagggccc ttgtctccag tagcattggg gtgcagatga gaagaataac agctcctctt 4440

cctcttctgc agaaagagct gacagatgag ttccgggagc tgcgggccac agtggagcgg 4500

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ctggatggtg cagcctggct caccctttgg gtctttggga cgtccttttt gcccttcctc 4620

ctctgtgcgg tgctgctcag tgcagttcag gtgagagcct ttggcttgtc aagtgcacag 4680

caatgctcag catccctg 4698

<210> SEQ ID NO 35
<211> LENGTH: 990
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 35

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acctgggacg aggtggccca gcgctcaggg tgcgaggagc ggtggctagt gatcgaccgt 120

-continued

aaggtgtaca acatcagcga gttcacccgc cggcatccag ggggctcccg ggtcatcagc 180

cactacgccg ggcaggatgc cacggatccc tttgtggcct tccacatcaa caagggcctt 240

gtgaagaagt atatgaactc tctcctgatt ggagaactgt ctccagagca gcccagcttt 300

gagcccacca agaataaaga gctgacagat gagttccggg agctgcgggc cacagtggag 360

cggatggggc tcatgaaggc caaccatgtc ttcttcctgc tgtacctgct gcacatcttg 420

ctgctggatg gtgcagcctg gctcaccctt tgggtctttg ggacgtcctt tttgcccttc 480

ctcctctgtg cggtgctgct cagtgcagtt caggcccagg ctggctggct gcagcatgac 540

tttgggcacc tgtcggtctt cagcacctca aagtggaacc atctgctaca tcattttgtg 600

attggccacc tgaagggggc ccccgccagt tggtggaacc acatgcactt ccagcaccat 660

gccaagccca actgcttccg caaagaccca gacatcaaca tgcatccctt cttctttgcc 720

ttggggaaga tcctctctgt ggagcttggg aaacagaaga aaaaatatat gccgtacaac 780

caccagcaca aatacttctt cctaattggg cccccagcct tgctgcctct ctacttccag 840

tggtatattt tctattttgt tatccagcga cccccagcct tgctgcctct ctacttccag 900

tggtatattt tctattttgt tatccagcga aagaagtggg tggacttggc ctggatgatt 960

accttctacg tccgcttctt cctcacttat 990

<210> SEQ ID NO 36
<211> LENGTH: 960
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 36

ggcccggcgc gcggcgtcgc caggccagct atggcccccg acccggtggc cgccgagacc 60

gcggctcagg gacctacccc gcgctacttc acctgggacg aggtggccca gcgctcaggg 120

tgcgaggagc ggtggctagt gatcgaccgt aaggtgtaca acatcagcga gttcacccgc 180

cggcatccag ggggctcccg ggtcatcagc cactacgccg ggcaggatgc cacggatccc 240

tttgtggcct tccacatcaa caagggcctt gtgaagaagt atatgaactc tctcctgatt 300

ggagaactgt ctccagagca gcccagcttt gagcccacca agaataaaga gctgacagat 360

gagttccggg agctgcgggc cacagtggag cggatggggc tcatgaaggc caaccatgtc 420

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tgggtctttg ggacgtcctt tttgcccttc ctcctctgtg cggtgctgct cagtgcagtt 540

caggcccagg ctggctggct gcagcatgac tttgggcacc tgtcggtctt cagcacctca 600

aagtggaacc atctgctaca tcattttgtg attggccacc tgaagggggc ccccgccagt 660

tggtggaacc acatgcactt ccagcaccat gccaagccca actgcttccg caaagaccca 720

gacatcaaca tgcatccctt cttctttgcc ttggggaaga tcctctctgt ggagcttggg 780

aaacagaaga aaaaatatat gccgtacaac caccagcaca aatacttctt cctaattggg 840

cccccagcct tgctgcctct ctacttccag tggtatattt tctattttgt tatccagcga 900

aagaagtggg tggacttggc ctggatcagc aaacaggaat acgatgaagc cgggcttcca 960

<210> SEQ ID NO 37
<211> LENGTH: 473
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 37

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tcacttatgt gccactattg gggctgaaag ccttcctggg ccttttcttc atagtcaggt 120

tcctggaaag caactggttt gtgtgggtga cacagatgaa ccatattccc atgcacattg 180

atcatgaccg gaacatggac tgggtttcca cccagctcct ggccacatgc aatgtccaca 240

agtctgcctt caatgactgg ttcagtggac acctcaactt ccagattgag caccatcttt 300

ttcccacgat gcctcgacac aattaccaca aagtggctcc cctggtgcag tccttgtgtg 360

ccaagcgtgg catagagtac cagtccaagc ccctgctgtc agccttcgcc gacatcatcc 420

actcactaaa ggagtcaggg cagctctggc tagatgccta tcttcaccaa taa 473

<210> SEQ ID NO 38
<211> LENGTH: 449
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5)...(5)
<223> OTHER INFORMATION: k = g or t/u at position 5
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)...(6)
<223> OTHER INFORMATION: m = a or c at position 6

<400> SEQUENCE: 38

gaatkmttac cttctacgtc cgcttcttcc tcacttatgt gccactattg gggctgaaag 60

cttcctgggc cttttcttca tagtcaggtt cctggaaagc aactggtttg tgtgggtgac 120

acagatgaac catattccca tgcacattga tcatgaccgg aacatggact gggtttccac 180

ccagctccag gccacatgca atgtccacaa gtctgccttc aatgactggt tcagtggaca 240

cctcaacttc cagattgagc accatctttt tcccacgatg cctcgacaca attaccacaa 300

agtggctccc ctggtgcagt ccttgtgtgc caagcatggc atagagtacc agtccaagcc 360

cctgctgtca gccttcgccg acatcatcca ctcactaaag gagtcagggc agctctggct 420

agatgcctat cttcaccaat aacaacagc 449

<210> SEQ ID NO 39
<211> LENGTH: 445
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (445)...(445)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 445

<400> SEQUENCE: 39

Met Ala Pro Asp Pro Val Ala Ala Glu Thr Ala Ala Gln Gly Pro Thr
1 5 10 15

Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln Arg Ser Gly Cys Glu
20 25 30

Glu Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn Ile Ser Glu Phe
35 40 45

Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser His Tyr Ala Gly
50 55 60

Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile Asn Lys Gly Leu
65 70 75 80

Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu Leu Ser Pro Glu
85 90 95

Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe
100 105 110

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Arg Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn
        115                 120                 125

His Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly
    130                 135                 140

Ala Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe
145                 150                 155                 160

Leu Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala Gln Ala Gly Trp
                165                 170                 175

Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr Ser Lys Trp
            180                 185                 190

Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys Gly Ala Pro
        195                 200                 205

Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala Lys Pro Asn
    210                 215                 220

Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe Phe Phe Ala
225                 230                 235                 240

Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys Lys Lys Tyr
                245                 250                 255

Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile Gly Pro Pro
            260                 265                 270

Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr Phe Val Ile
        275                 280                 285

Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr Phe Tyr Val
    290                 295                 300

Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys Ala Phe Leu
305                 310                 315                 320

Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp Phe Val Trp
                325                 330                 335

Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His Asp Arg Asn
            340                 345                 350

Met Asp Trp Val Ser Thr Gln Leu Leu Ala Thr Cys Asn Val His Lys
        355                 360                 365

Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
    370                 375                 380

His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His Lys Val Ala
385                 390                 395                 400

Pro Leu Val Gln Ser Leu Cys Ala Lys Arg Gly Ile Glu Tyr Gln Ser
                405                 410                 415

Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser Leu Lys Glu
            420                 425                 430

Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln Xaa
        435                 440                 445


<210> SEQ ID NO 40
<211> LENGTH: 465
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (432)...(432)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 432
<221> NAME/KEY: VARIANT
<222> LOCATION: (458)...(458)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 458

<400> SEQUENCE: 40

Gln Gly Pro Thr Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln Arg
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 1               5                  10                  15

Ser Gly Cys Glu Glu Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn
            20                  25                  30

Ile Ser Glu Phe Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser
        35                  40                  45

His Tyr Ala Gly Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile
    50                  55                  60

Asn Lys Gly Leu Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu
65                  70                  75                  80

Leu Ser Pro Glu Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu
                85                  90                  95

Thr Asp Glu Phe Arg Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu
            100                 105                 110

Met Lys Ala Asn His Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu
        115                 120                 125

Leu Leu Asp Gly Ala Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser
    130                 135                 140

Phe Leu Pro Phe Leu Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala
145                 150                 155                 160

Gln Ala Gly Trp Leu Gln His Asp Tyr Gly His Leu Ser Val Tyr Arg
                165                 170                 175

Lys Pro Lys Trp Asn His Leu Val His Lys Phe Val Ile Gly His Leu
            180                 185                 190

Lys Gly Ala Ser Ala Asn Trp Trp Asn His Arg His Phe Gln His His
        195                 200                 205

Ala Lys Pro Asn Ile Phe His Lys Asp Pro Asp Val Asn Met Leu His
    210                 215                 220

Val Phe Val Leu Gly Glu Trp Gln Pro Ile Glu Tyr Gly Lys Lys Lys
225                 230                 235                 240

Leu Lys Tyr Leu Pro Tyr Asn His Gln His Glu Tyr Phe Phe Leu Ile
                245                 250                 255

Gly Pro Pro Leu Leu Ile Pro Met Tyr Phe Gln Tyr Gln Ile Ile Met
            260                 265                 270

Thr Met Ile Val His Lys Asn Trp Val Asp Leu Ala Trp Ala Val Ser
        275                 280                 285

Tyr Tyr Ile Arg Phe Phe Ile Thr Tyr Ile Pro Phe Tyr Gly Ile Leu
    290                 295                 300

Gly Ala Leu Leu Phe Leu Asn Phe Ile Arg Phe Leu Glu Ser His Trp
305                 310                 315                 320

Phe Val Trp Val Thr Gln Met Asn His Ile Val Met Glu Ile Asp Gln
                325                 330                 335

Glu Ala Tyr Arg Asp Trp Phe Ser Ser Gln Leu Thr Ala Thr Cys Asn
            340                 345                 350

Val Glu Gln Ser Phe Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe
        355                 360                 365

Gln Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Leu His
    370                 375                 380

Lys Ile Ala Pro Leu Val Lys Ser Leu Cys Ala Lys His Gly Ile Glu
385                 390                 395                 400

Tyr Gln Glu Lys Pro Leu Leu Arg Ala Leu Leu Asp Ile Ile Arg Ser
                405                 410                 415

Leu Lys Lys Ser Gly Lys Leu Trp Leu Asp Ala Tyr Leu His Lys Xaa
            420                 425                 430
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Ser His Ser Pro Arg Asp Thr Val Gly Lys Gly Cys Arg Trp Gly Asp
435 440 445

Gly Gln Arg Asn Asp Gly Leu Leu Phe Xaa Gly Val Ser Glu Arg Leu
450 455 460

Val
465

<210> SEQ ID NO 41
<211> LENGTH: 360
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 41

Met Ala Pro Asp Pro Val Ala Ala Glu Thr Ala Ala Gln Gly Pro Thr
1 5 10 15

Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln Arg Ser Gly Cys Glu
20 25 30

Glu Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn Ile Ser Glu Phe
35 40 45

Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser His Tyr Ala Gly
50 55 60

Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile Asn Lys Gly Leu
65 70 75 80

Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu Leu Ser Pro Glu
85 90 95

Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe
100 105 110

Arg Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn
115 120 125

His Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly
130 135 140

Ala Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe
145 150 155 160

Leu Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala Gln Ala Gly Trp
165 170 175

Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr Ser Lys Trp
180 185 190

Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys Gly Ala Pro
195 200 205

Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala Lys Pro Asn
210 215 220

Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe Phe Phe Ala
225 230 235 240

Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys Lys Lys Tyr
245 250 255

Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile Gly Pro Pro
260 265 270

Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr Phe Val Ile
275 280 285

Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr Phe Tyr Val
290 295 300

Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys Ala Phe Leu
305 310 315 320

Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp Phe Val Trp

-continued

325 330 335

Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His Asp Arg Asn
340 345 350

Met Asp Trp Val Ser Thr Gln Leu
355 360

<210> SEQ ID NO 42
<211> LENGTH: 347
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (251)...(251)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 251
<221> NAME/KEY: VARIANT
<222> LOCATION: (329)...(330)
<223> OTHER INFORMATION: Xaa = Unknown or other at these positions

<400> SEQUENCE: 42

Gln Gly Pro Thr Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln Arg
1 5 10 15

Ser Gly Cys Glu Glu Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn
20 25 30

Ile Ser Glu Phe Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser
35 40 45

His Tyr Ala Gly Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile
50 55 60

Asn Lys Gly Leu Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu
65 70 75 80

Leu Ser Pro Glu Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu
85 90 95

Thr Asp Glu Phe Arg Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu
100 105 110

Met Lys Ala Asn His Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu
115 120 125

Leu Leu Asp Gly Ala Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser
130 135 140

Phe Leu Pro Phe Leu Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala
145 150 155 160

Gln Ala Gly Trp Leu Gln His Asp Gly His Leu Ser Val Phe Ser Thr
165 170 175

Ser Lys Trp Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys
180 185 190

Gly Ala Pro Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala
195 200 205

Lys Pro Asn Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe
210 215 220

Phe Phe Ala Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys
225 230 235 240

Lys Lys Tyr Met Pro Tyr Asn His Gln His Xaa Tyr Phe Phe Leu Ile
245 250 255

Gly Pro Pro Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr
260 265 270

Phe Val Ile Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Ile Ser Lys
275 280 285

Gln Glu Tyr Asp Glu Ala Gly Leu Pro Leu Ser Thr Ala Asn Ala Ser
290 295 300

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Lys Arg Asp Leu Pro Arg Ala Thr Ser Pro Gly Thr Arg Trp Pro Ser
305                 310                 315                 320

Ala Gln Gly Ala Arg Ser Gly Gly Xaa Xaa Ser Thr Val Arg Cys Thr
                325                 330                 335

Thr Ser Ala Ser Ser Pro Ala Gly Ile Gln Gly
            340                 345


<210> SEQ ID NO 43
<211> LENGTH: 444
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (444)...(444)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 444

<400> SEQUENCE: 43

Met Ala Pro Asp Pro Val Ala Ala Glu Thr Ala Ala Gln Gly Pro Thr
 1               5                  10                  15

Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln Arg Ser Gly Cys Glu
            20                  25                  30

Glu Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn Ile Ser Glu Phe
        35                  40                  45

Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser His Tyr Ala Gly
    50                  55                  60

Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile Asn Lys Gly Leu
65                  70                  75                  80

Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu Leu Ser Pro Glu
                85                  90                  95

Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe
            100                 105                 110

Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn His
        115                 120                 125

Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly Ala
    130                 135                 140

Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe Leu
145                 150                 155                 160

Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala Gln Ala Gly Trp Leu
                165                 170                 175

Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr Ser Lys Trp Asn
            180                 185                 190

His Leu Leu His His Phe Val Ile Gly His Leu Lys Gly Ala Pro Ala
        195                 200                 205

Ser Trp Trp Asn His Met His Phe Gln His His Ala Lys Pro Asn Cys
    210                 215                 220

Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe Phe Phe Ala Leu
225                 230                 235                 240

Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys Lys Lys Tyr Met
                245                 250                 255

Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile Gly Pro Pro Ala
            260                 265                 270

Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr Phe Val Ile Gln
        275                 280                 285

Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr Phe Tyr Val Arg
    290                 295                 300
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Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys Ala Phe Leu Gly
305                 310                 315                 320

Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp Phe Val Trp Val
                325                 330                 335

Thr Gln Met Asn His Ile Pro Met His Ile Asp His Asp Arg Asn Met
            340                 345                 350

Asp Trp Val Ser Thr Gln Leu Leu Ala Thr Cys Asn Val His Lys Ser
        355                 360                 365

Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu His
    370                 375                 380

His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His Lys Val Ala Pro
385                 390                 395                 400

Leu Val Gln Ser Leu Cys Ala Lys Arg Gly Ile Glu Tyr Gln Ser Lys
                405                 410                 415

Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser Leu Lys Glu Ser
            420                 425                 430

Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln Xaa
        435                 440


<210> SEQ ID NO 44
<211> LENGTH: 458
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (458)...(458)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 458

<400> SEQUENCE: 44

Met Ala Ala Ala Pro Ser Val Arg Thr Phe Thr Arg Ala Glu Val Leu
 1               5                  10                  15

Asn Ala Glu Ala Leu Asn Glu Gly Lys Lys Asp Ala Glu Ala Pro Phe
            20                  25                  30

Leu Met Ile Ile Asp Asn Lys Val Tyr Asp Val Arg Glu Phe Val Pro
        35                  40                  45

Asp His Pro Gly Gly Ser Val Ile Leu Thr His Val Gly Lys Asp Gly
    50                  55                  60

Thr Asp Val Phe Asp Thr Phe His Pro Glu Ala Ala Trp Glu Thr Leu
65                  70                  75                  80

Ala Asn Phe Tyr Val Gly Asp Ile Asp Glu Ser Asp Arg Asp Ile Lys
                85                  90                  95

Asn Asp Asp Phe Ala Ala Glu Val Arg Lys Leu Arg Thr Leu Phe Gln
            100                 105                 110

Ser Leu Gly Tyr Tyr Asp Ser Ser Lys Ala Tyr Tyr Ala Phe Lys Val
        115                 120                 125

Ser Phe Asn Leu Cys Ile Trp Gly Leu Ser Thr Val Ile Val Ala Lys
    130                 135                 140

Trp Gly Gln Thr Ser Thr Leu Ala Asn Val Leu Ser Ala Ala Leu Leu
145                 150                 155                 160

Gly Leu Phe Trp Gln Gln Cys Gly Trp Leu Ala His Asp Phe Leu His
                165                 170                 175

His Gln Val Phe Gln Asp Arg Phe Trp Gly Asp Leu Phe Gly Ala Phe
            180                 185                 190

Leu Gly Gly Val Cys Gln Gly Phe Ser Ser Ser Trp Trp Lys Asp Lys
        195                 200                 205

His Asn Thr His His Ala Ala Pro Asn Val His Gly Glu Asp Pro Asp
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-continued

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    210                 215                 220

Ile Asp Thr His Pro Leu Leu Thr Trp Ser Glu His Ala Leu Glu Met
225                 230                 235                 240

Phe Ser Asp Val Pro Asp Glu Glu Leu Thr Arg Met Trp Ser Arg Phe
                245                 250                 255

Met Val Leu Asn Gln Thr Trp Phe Tyr Phe Pro Ile Leu Ser Phe Ala
            260                 265                 270

Arg Leu Ser Trp Cys Leu Gln Ser Ile Leu Phe Val Leu Pro Asn Gly
        275                 280                 285

Gln Ala His Lys Pro Ser Gly Ala Arg Val Pro Ile Ser Leu Val Glu
    290                 295                 300

Gln Leu Ser Leu Ala Met His Trp Thr Trp Tyr Leu Ala Thr Met Phe
305                 310                 315                 320

Leu Phe Ile Lys Asp Pro Val Asn Met Leu Val Tyr Phe Leu Val Ser
                325                 330                 335

Gln Ala Val Cys Gly Asn Leu Leu Ala Ile Val Phe Ser Leu Asn His
            340                 345                 350

Asn Gly Met Pro Val Ile Ser Lys Glu Glu Ala Val Asp Met Asp Phe
        355                 360                 365

Phe Thr Lys Gln Ile Ile Thr Gly Arg Asp Val His Pro Gly Leu Phe
    370                 375                 380

Ala Asn Trp Phe Thr Gly Gly Leu Asn Tyr Gln Ile Glu His His Leu
385                 390                 395                 400

Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro Ala Val
                405                 410                 415

Glu Thr Leu Cys Lys Lys Tyr Asn Val Arg Tyr His Thr Thr Gly Met
            420                 425                 430

Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val Ser Lys
        435                 440                 445

Ala Ala Ser Lys Met Gly Lys Ala Gln Xaa
    450                 455


<210> SEQ ID NO 45
<211> LENGTH: 445
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (445)...(445)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 445

<400> SEQUENCE: 45

Met Ala Pro Asp Pro Val Ala Ala Glu Thr Ala Ala Gln Gly Pro Thr
 1               5                  10                  15

Pro Arg Tyr Phe Thr Trp Asp Glu Val Ala Gln Arg Ser Gly Cys Glu
            20                  25                  30

Glu Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn Ile Ser Glu Phe
        35                  40                  45

Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser His Tyr Ala Gly
    50                  55                  60

Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile Asn Lys Gly Leu
65                  70                  75                  80

Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu Leu Ser Pro Glu
                85                  90                  95

Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe
            100                 105                 110
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```
Arg Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn
        115                 120                 125

His Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly
    130                 135                 140

Ala Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe
145                 150                 155                 160

Leu Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala Gln Ala Gly Trp
                165                 170                 175

Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr Ser Lys Trp
            180                 185                 190

Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys Gly Ala Pro
        195                 200                 205

Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala Lys Pro Asn
    210                 215                 220

Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe Phe Phe Ala
225                 230                 235                 240

Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys Lys Lys Tyr
                245                 250                 255

Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile Gly Pro Pro
            260                 265                 270

Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr Phe Val Ile
        275                 280                 285

Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr Phe Tyr Val
    290                 295                 300

Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys Ala Phe Leu
305                 310                 315                 320

Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp Phe Val Trp
                325                 330                 335

Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His Asp Arg Asn
            340                 345                 350

Met Asp Trp Val Ser Thr Gln Leu Leu Ala Thr Cys Asn Val His Lys
        355                 360                 365

Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
    370                 375                 380

His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His Lys Val Ala
385                 390                 395                 400

Pro Leu Val Gln Ser Leu Cys Ala Lys Arg Gly Ile Glu Tyr Gln Ser
                405                 410                 415

Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser Leu Lys Glu
            420                 425                 430

Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln Xaa
        435                 440                 445


<210> SEQ ID NO 46
<211> LENGTH: 447
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: (447)...(447)
<223> OTHER INFORMATION: Xaa = Unknown or other at position 447

<400> SEQUENCE: 46

Met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala
 1               5                  10                  15
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```
His Asn Thr Lys Asp Asp Leu Leu Leu Ala Ile Arg Gly Arg Val Tyr
            20                  25                  30

Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Gly Val Asp Thr Leu
        35                  40                  45

Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val Phe Glu Met Tyr His
    50                  55                  60

Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr
65                  70                  75                  80

Leu Val Ser Asn Glu Leu Pro Ile Phe Pro Glu Pro Thr Val Phe His
                85                  90                  95

Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asn Ile
            100                 105                 110

Asp Pro Lys Asn Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe
        115                 120                 125

Gly Ser Leu Ile Ala Ser Tyr Tyr Ala Gln Leu Phe Val Pro Phe Val
    130                 135                 140

Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe
145                 150                 155                 160

Ala Cys Ala Gln Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe
                165                 170                 175

Ser Val Thr His Asn Pro Thr Val Trp Lys Ile Leu Gly Ala Thr His
            180                 185                 190

Asp Phe Phe Asn Gly Ala Ser Tyr Leu Val Trp Met Tyr Gln His Met
        195                 200                 205

Leu Gly His His Pro Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val
    210                 215                 220

Ser Thr Ser Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp
225                 230                 235                 240

Phe Val Asn His Ile Asn Gln His Met Phe Val Pro Phe Leu Tyr Gly
                245                 250                 255

Leu Leu Ala Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe
            260                 265                 270

Val Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His
        275                 280                 285

Thr Val Met Phe Trp Gly Gly Lys Ala Phe Phe Val Trp Tyr Arg Leu
    290                 295                 300

Ile Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe
305                 310                 315                 320

Thr Val Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln
                325                 330                 335

Ala Asn His Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn
            340                 345                 350

Gly Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln
        355                 360                 365

Asp Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu
    370                 375                 380

Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His
385                 390                 395                 400

Tyr Pro Asp Ile Leu Ala Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys
                405                 410                 415

Val Pro Tyr Leu Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His
            420                 425                 430

Leu Glu His Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu Xaa
```

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435 440 445

<210> SEQ ID NO 47
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Upstream Primer RO676

<400> SEQUENCE: 47

atacgtgaat tcgccgccac catggccccc gacccggtg 39

<210> SEQ ID NO 48
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Downstream Primer RO677

<400> SEQUENCE: 48

tatccgctcg agttattggt gaagataggc atctag 36

<210> SEQ ID NO 49
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Vector Primer RO329

<400> SEQUENCE: 49

cagaccaact ggtaatggta g 21

<210> SEQ ID NO 50
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Vector Primer RO384

<400> SEQUENCE: 50

tcaggcccaa gctggatggc tgcaacatg 29

<210> SEQ ID NO 51
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Vector Primer RO328

<400> SEQUENCE: 51

ctcctggagc ccgtcagtat c 21

<210> SEQ ID NO 52
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Vector Primer RO388

<400> SEQUENCE: 52

atggtgggga agaggtggtg ctcaatctg 29

<210> SEQ ID NO 53
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

-continued

<220> FEATURE:
<223> OTHER INFORMATION: Primer RO430

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What is claimed is:

1. A method for producing a polyunsaturated fatty acid comprising the steps of:

a) isolating said nucleotide sequence represented by SEQ ID NO:1 (FIG. 12);

b) constructing a vector comprising said isolated nucleotide sequence;

c) introducing said vector into a host cell under time and conditions sufficient for expression of said human Δ5-desaturase enzyme; and

d) exposing said expressed human Δ5-desaturase enzyme to a substrate polyunsaturated fatty acid in order to convert said substrate to a product polyunsaturated fatty acid.

2. The method according to claim 1, wherein said substrate polyunsaturated fatty acid is dihomo-γ-linolenic acid (DGLA) or 20:4n-3 and said product polyunsaturated fatty acid is arachidonic acid (AA) or eicosapentaenoic acid (EPA), respectively.

3. The method according to claim 1 further comprising the step of exposing said product polyunsaturated fatty acid to an elongase in order to convert said product polyunsaturated fatty acid to another polyunsaturated fatty acid.

4. The method according to claim 3 wherein said product polyunsaturated fatty acid is AA or EPA and said another polyunsaturated fatty acid is adrenic acid or (n-3)-docosapentaenoic acid, respectively.

5. The method of claim 3 further comprising the steps of exposing said another polyunsaturated fatty acid to an additional desaturase in order to convert said another polyunsaturated fatty acid to a final polyunsaturated fatty acid.

6. The method of claim 5 wherein said final polyunsaturated fatty acid is (n-6)-docosapentaenoic acid or docosahexaenoic (DHA) acid.

* * * * *

2

Tyrosine hydroxylase: human isoforms, structure and regulation in physiology and pathology

Toshiharu Nagatsu

Institute for Comprehensive Medical Science, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1, Japan

Introduction

Tyrosine 3-hydroxylase (TH; EC 1.14.16.2) catalyses the first step in the biosynthesis of catecholamines (dopamine, noradrenaline and adrenaline)[1]. Catecholamines function as neurotransmitters in dopamine, noradrenaline and adrenaline neurons in the brain and retina, and in peripheral sympathetic noradrenaline neurons, and also as hormones (adrenaline and noradrenaline) in the adrenal medulla. Catecholamine neurotransmitters in the brain regulate a wide range of high-level brain functions, such as movement, emotion, learning, memory, biorhythm, reproduction and endocrine function, by acting across synapses through dopamine receptors and α- and β-adrenaline receptors of the neuronal network. In peripheral tissues, noradrenergic sympathetic neurons distributed in organs secrete noradrenaline as a neurotransmitter from their nerve endings. The adrenomedullary cells secrete adrenaline (and a small amount of noradrenaline) into the blood as hormones which regulate various functions indispensable to the maintenance of life, e.g. autonomic function, stress reactions, blood glucose level, blood pressure and blood circulation, by acting on cells with α- and β-adrenaline receptors.

TH plays important roles in physiology and pathology through the regulation of catecholamine biosynthesis. Catecholamines are known to be

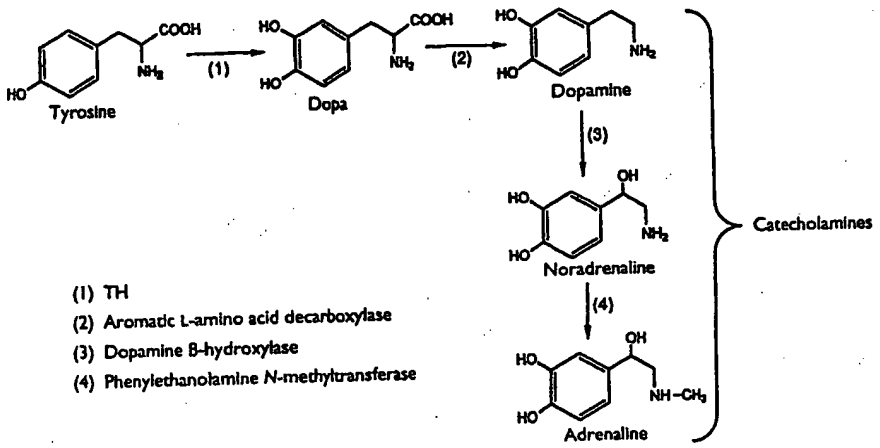


Figure 1. Pathway of biosynthesis of catecholamines (dopamine, noradrenaline and adrenaline) from tyrosine, and the catecholamine-synthesizing enzymes

involved in many diseases, including neuropsychiatric diseases (Parkinson's disease, affective disorders or manic depressive illness, schizophrenia, etc.); cardiovascular diseases (hypertension, cardiac diseases, etc.); and metabolic diseases (diabetes mellitus, etc.).

Catecholamines are synthesized from L-tyrosine by the pathway shown in Figure 1. Thus dopaminergic neurons contain the following synthesizing enzymes: (1) TH and (2) aromatic L-amino acid decarboxylase (EC 4.1.1.28; also known as dopa decarboxylase). Noradrenergic neurons or adrenomedullary cells have a third synthesizing enzyme: (3) dopamine β-hydroxylase (EC 1.14.17.1; also known as dopamine β-monooxygenase). Cells that synthesize adrenaline also have a fourth synthesizing enzyme in addition to these three: (4) phenylethanolamine *N*-methyltransferase (EC 2.1.1.28; also known as noradrenaline *N*-methyltransferase).

TH was discovered in 1964[1]. At that time, of the four enzymes involved in catecholamine biosynthesis, only the enzyme responsible for converting tyrosine to dopa was elusive. Tyrosinase was assumed to catalyse the reaction, but was not found in catecholamine-containing tissues, including the brain. Others had suggested that dopa formation was non-enzymic *in vivo*, since it could be observed easily under various conditions *in vitro*. TH activity was first detected with a newly developed, sensitive radio-isotopic assay which used L-[^{14}C]-tyrosine as substrate. L-[^{14}C]-dopa, enzymically formed, was isolated on an alumina column and assayed; however, when D-[^{14}C]-tyrosine was used as a control, no radiolabelled dopa was formed. This evidence clearly demonstrated that an enzyme, such as TH, catalyses the conversion of L-tyrosine to L-dopa. TH was later found to be the rate-limiting enzyme in the biosynthesis of catecholamines[2].

TH requires a pteridine and ferrous ion as essential cofactors[1]. The natural tetrahydropteridine cofactor, tetrahydrobiopterin, was found to be most active[3]. The enzyme requires molecular oxygen as a substrate and is therefore a monooxygenase (also known as tyrosine 3-monooxygenase)[1].

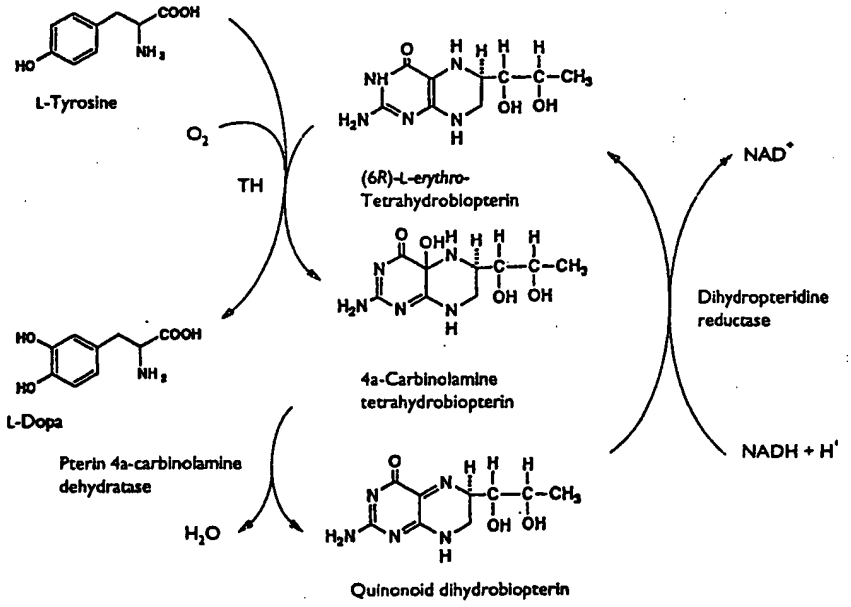
The purification of TH was difficult, but was finally achieved in early 1980. By 1990, complex regulatory mechanisms had been found, including feedback regulation by catecholamines, and activation or deactivation due to phosphorylation by protein kinases or dephosphorylation by phosphatases. Since 1985, the structure of TH from various species, including humans, has been determined by cDNA cloning.

Properties of TH as a pteridine-dependent monooxygenase

TH is expressed in the catecholamine neurons that are present in discrete regions of the brain and retina, in the noradrenaline neurons of sympathetic ganglia and sympathetic nerves, and in adrenaline and noradrenaline cells of the adrenal medulla.

The reaction of TH is considered to be similar to that of phenylalanine 4-hydroxylase (EC 1.14.16.1)[4] (Figure 2). The TH substrates L-tyrosine and molecular oxygen and the tetrahydrobiopterin natural cofactor are converted to L-dopa and 4a-carbinolamine tetrahydrobiopterin. 4a-Carbinolamine tetrahydrobiopterin is converted to quinonoid dihydrobiopterin by pterin 4a-carbinolamine dehydratase (EC 4.2.1.96). Quinonoid dihydrobiopterin is

Figure 2. Reaction catalysed by TH in relation to the tetrahydrobiopterin cofactor



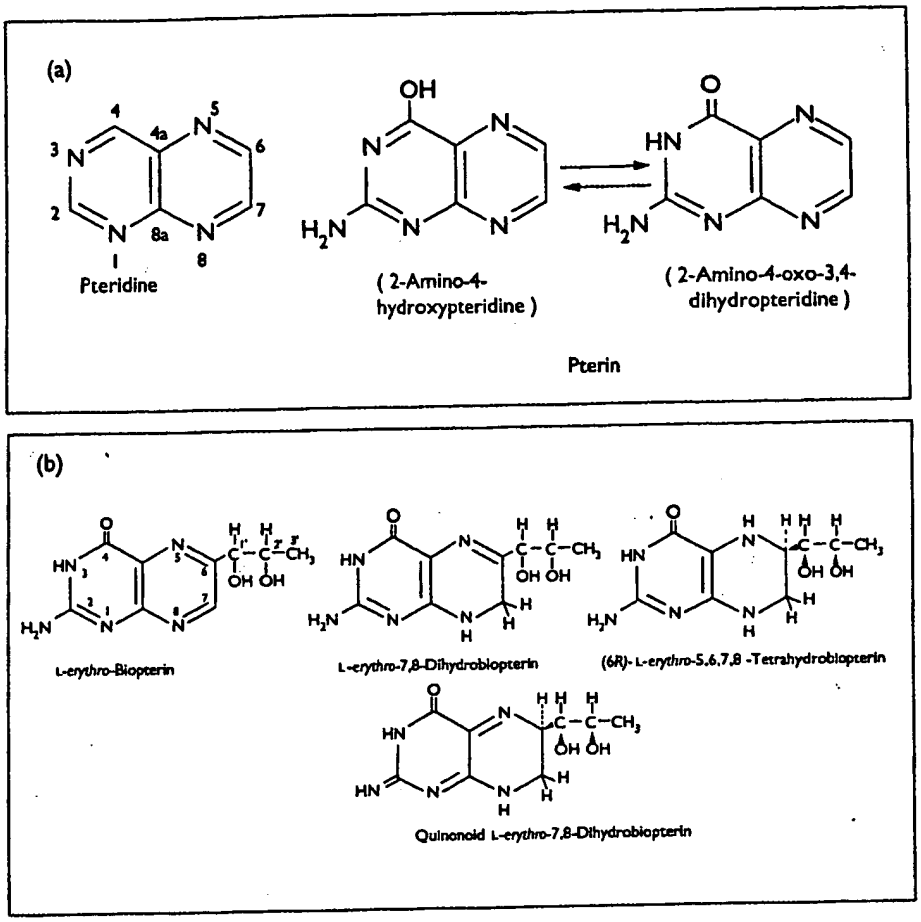


Figure 3. (a) Structure of pteridine and pterin; (b) structure of four forms of tetrahydrobiopterin

reduced back to tetrahydrobiopterin by dihydropteridine reductase (EC 1.6.99.7) with NADH as the cofactor.

The term pterin, which was originally used to describe a factor in the pigments of butterfly wings, is now used for the natural pteridine compounds, most of which have the structure of 2-amino-4-hydroxypteridine. *In vivo*, pterin has the structure of the oxoform, 2-amino-4-oxo-3,4-dihydropteridine (Figure 3a). The natural form of the tetrahydropteridine cofactor, L-*erythro*-tetrahydrobiopterin, was first discovered as the cofactor of phenylalanine 4-hydroxylase (Figure 3b). The stereochemical structure is the (6*R*)-form in the reduced tetrahydro- form. Enzymically produced quinonoid dihydrobiopterin is also spontaneously and rapidly converted to 7,8-dihydrobiopterin, and then further oxidized to biopterin. The tissue concentration of the latter two forms is low compared with the former two reduced forms.

Tetrahydrobiopterin has many important functions as the cofactor of pterin-requiring monooxygenases and also of nitric oxide synthase.

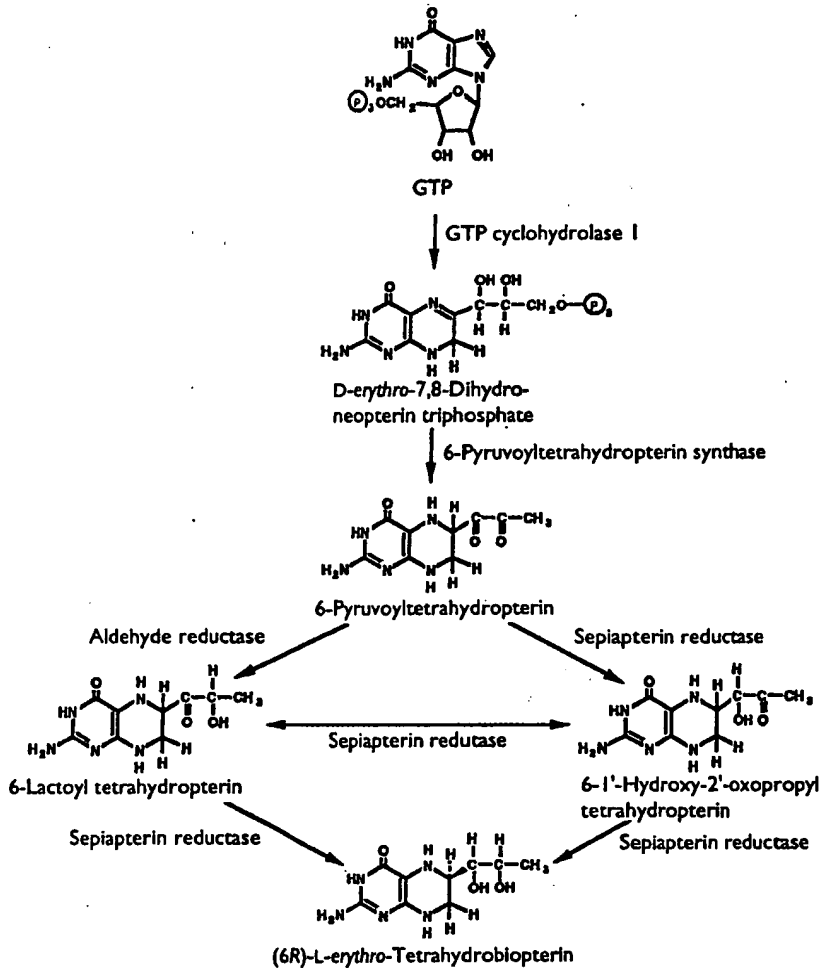


Figure 4. Pathway of biosynthesis of tetrahydrobiopterin, a cofactor for TH

Tetrahydrobiopterin is synthesized from GTP by the pathway shown in Figure 4. Three enzymes are required: (i) GTP cyclohydrolase I (EC 3.5.4.16); (ii) 6-pyruvoyltetrahydropterin synthase (EC 4.6.1.10); and (iii) sepiapterin reductase (EC 1.1.1.153). The third step may be catalysed by sepiapterin reductase alone or by aldehyde reductase (EC 1.1.1.21) and then by sepiapterin reductase (Figure 5). The concentration of tetrahydrobiopterin synthesized from GTP partly regulates the activity of TH.

TH also requires Fe^{2+} for activity. Human TH in crude tissue preparations is highly activated by exogenously added Fe^{2+}.

TH has been purified from bovine adrenal medulla[5], rat adrenals[6], rat pheochromocytoma[7], human adrenals and human brain[8]. In human adrenals and brain, TH is composed of both active and less active forms. The less active forms can be detected by enzyme immunoassay and Western blot analysis[7]. As

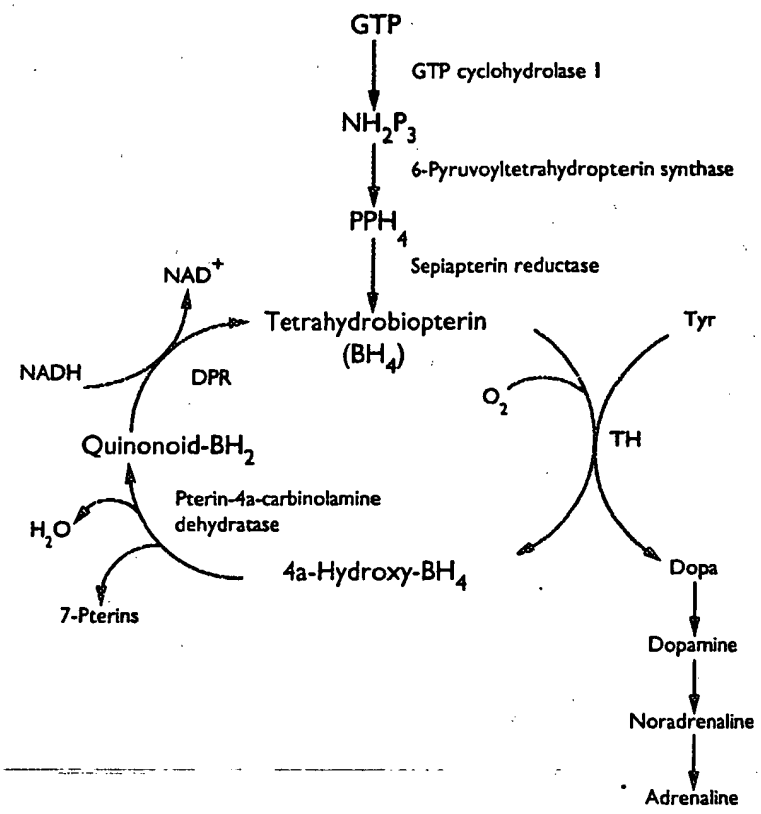


Figure 5. Relationship of tetrahydrobiopterin biosynthetic pathway to catecholamine biosynthesis via tyrosine hydroxylase
Abbreviations used: NH_2P_3, dihydroneopterin triphosphate; PPH_4, 6-pyruvoyltetrahydropterin; BH_4, tetrahydrobiopterin; BH_2, dihydroblopterin; DPR, dihydropteridine reductase.

described below, human TH has four types of mRNA encoding four isoform proteins.

Rat, bovine or human TH is a tetrameric protein of about 240 kDa, each subunit having a mass of approx. 60 kDa. Each subunit has a C-terminal catalytic domain that binds the substrates tyrosine and molecular oxygen, and the pterin cofactor, and an N-terminal regulatory domain containing phosphorylated serine residues.

Isoforms of human TH

Since it was difficult to obtain sufficient amounts of TH protein to elucidate the complete amino acid sequence, the primary structure of TH from various species including humans has been deduced from the nucleotide sequence of TH cDNA. A full-length cDNA containing the entire sequence of rat TH was first cloned from rat pheochromocytoma[9]. The open-reading frame, including

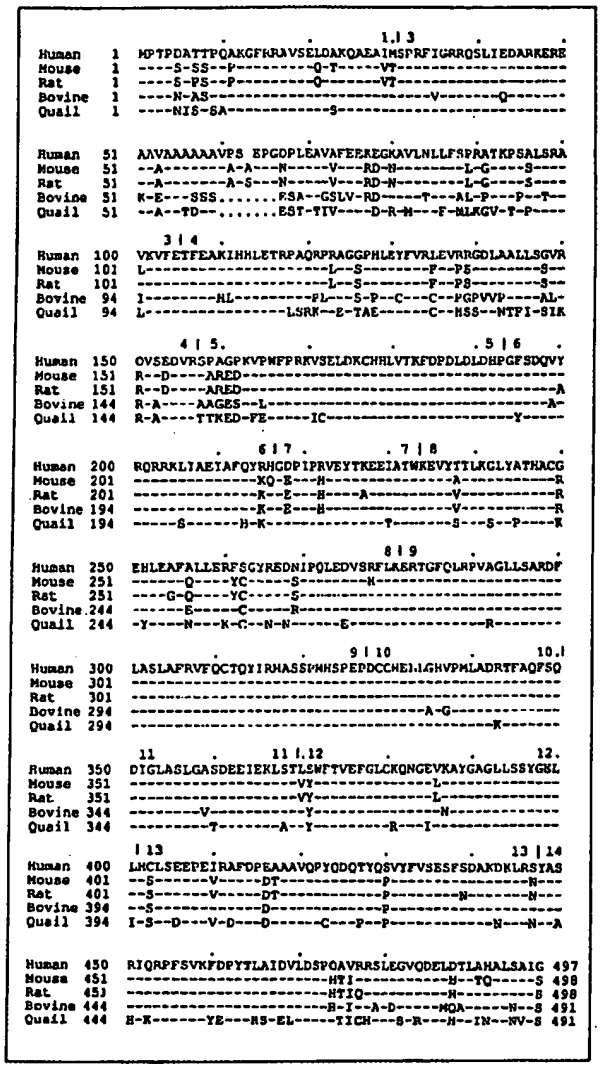


Figure 6. Comparison of the structures of human (type 1), mouse, rat, bovine and quail TH
Identical amino acids of mouse, rat, bovine and quail TH are expressed by hyphens. Vertical bars and the numbers above the human amino acid sequence represent break-point of exons and the exon numbers in the human TH gene, as shown in Figure 8.

the initiation codon, contains 1494 bp that encode 498 amino acids. Only one cDNA was cloned from rat, mouse, or bovine tissues. Figure 6 shows a comparison of amino acid sequences between human TH (type 1) and animal (mouse, rat, bovine and quail) TH. The sequence similarity of TH from various animals is high at the catalytic domain near the carboxyl region.

In contrast with a single TH cDNA in animals, human TH has four isoforms (hTH1–4) of mRNA encoding different proteins[10,11] (Figure 7). Nucleotide sequence analyses of full-length cDNA of types 1 and 2[10], type 3 and type 4[11] revealed that these four mRNAs differ only in the inclusion/exclusion of 12, 81 and 93 (12 plus 81) bp sequences, respectively, between nucleotides 90 and 91 of hTH1 mRNA. Since this insertion does not alter the reading frame of the protein-coding region, type-4 cDNA encodes the longest TH molecules. Southern blot analysis of human genomic DNA has

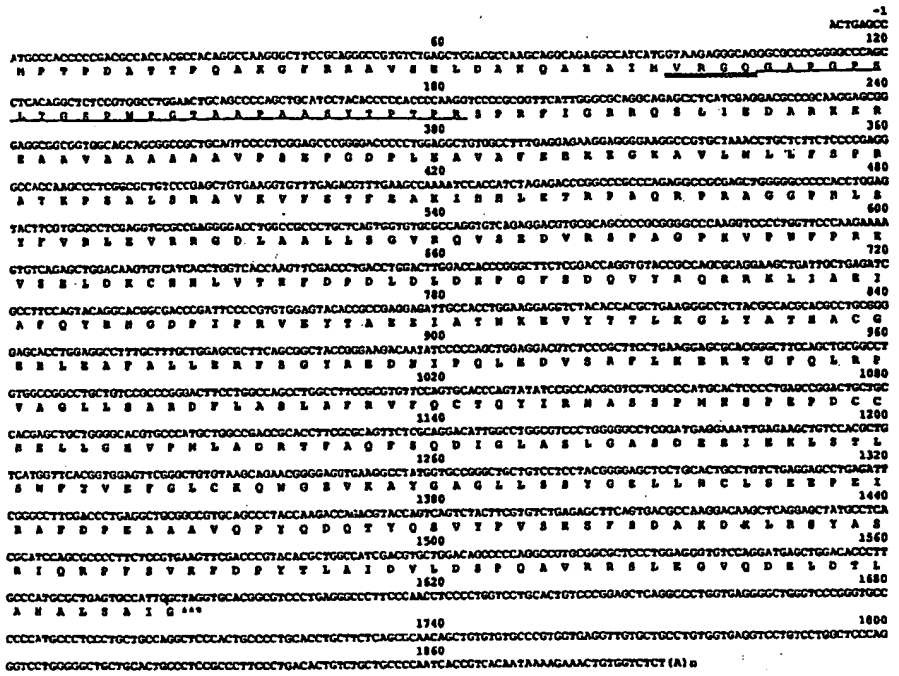


Figure 7. Nucleotide sequence and deduced amino acid sequence of hTH4 cDNA
The 81 bp sequence on the single line and the 12 bp sequence on the double line are deleted in hTH2 cDNA and hTH3 cDNA, respectively. The two sequences corresponding to the 93 bp are deleted in hTH1 cDNA, which is common among TH cDNA from various animals.

suggested that the human TH gene exists as a single gene per haploid DNA, indicating that these different human mRNAs are produced through alternative mRNA splicing from a single primary transcript[11].

Genomic clones encoding the human TH gene were isolated and characterized[12,13]. The human TH gene is composed of 14 exons, interrupted by 13 introns, spanning approximately 8.5 kb (Figure 8). The nucleotide sequence of the coding regions is the same as that of type-4 cDNA. The 12 bp insertion sequence is derived from the 3′-terminal portion of exon 1 (also called exon 1_2) and the 81-bp insertion sequence is encoded by exon 2 (also called exon 1_3). The N-terminal region is encoded by the 5′-portion of exon 1 (also called exon 1_1), and the remaining region from exon 3 to exon 14 (also called exon 2 to exon 13 for comparison with animal TH genes), is common to all four kinds of mRNA. Figure 8 summarizes the alternative splicing patterns which generate the four types of human TH mRNA. There are two modes of alternative splicing: (i) the alternate use of two donor sites in exon 1 (also called exon 1_1 and exon 1_2), whereby the selection of the two donor sites determines the insertion/deletion of the 12 bp sequence (also called exon 1_2); (ii) the other mode is the insertion/exclusion of an entire exon 2 (also called exon 1_3) that is specific for the human TH gene. Expression of type 1/2 or type 3/4 human TH

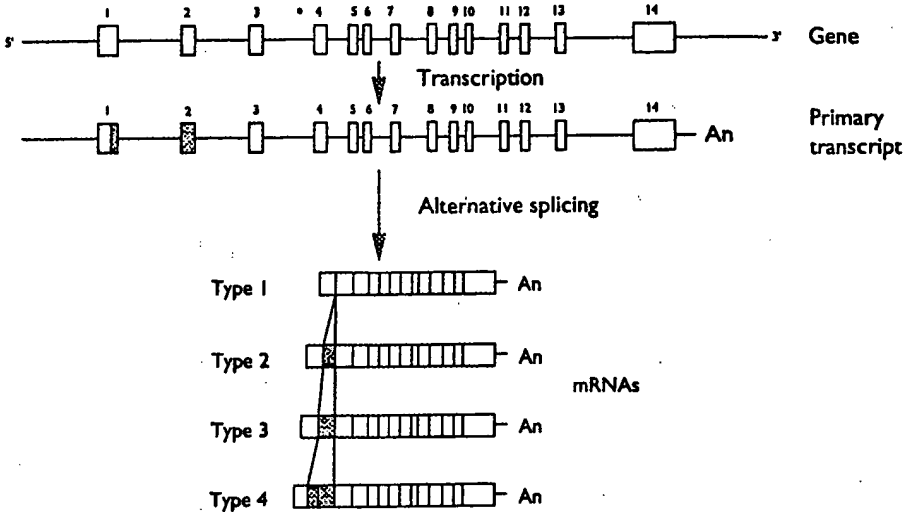


Figure 8. Structure of the human TH gene and schematic illustration of the alternative splicing pathway producing the four types of human TH mRNA from the primary transcript
The 3'-terminal, 12 bp sequence of exon 1 is also named exon 1_1, and the exon 2 is also named exon 1_3.

mRNA is determined by exclusion or inclusion of exon 2 (or exon 1_3) in the spliced products. The other 12 exons downstream from exon 3 (also called exon 2 for comparison with animal TH genes) are spliced and incorporated into mature mRNA.

hTH1 is similar to the enzyme from various animals. hTH1–4 have been expressed in COS cells, in *Xenopus* oocytes and in vertebrate cells. The expressed human TH types 1–4 show similar K_m values for tyrosine and the pteridine cofactor. However, the four types of human TH have different specific activities: hTH1 has the highest specific activity; the values for the other enzymes range from about 30% to 40% of that of hTH1.

hTH1–4 cDNAs have also been expressed in *Escherichia coli*, and large amounts of pure human TH have been obtained to characterize their properties[14,15].

mRNAs encoding the four isoforms of human TH have been detected in human neuroendocrine tissues and quantitatively determined in human brain (substantia nigra) using reverse transcription-polymerase chain reaction (RT-PCR). hTH1 and hTH2 are major species, and hTH3 and hTH4 are minor species. About 5% of the total human TH mRNA is represented by hTH3 and hTH4 in the normal human substantia nigra[16]. The approximate ratio of hTH1, hTH2, hTH3 and hTH4 mRNAs to the total amount of TH mRNA is 45:52:1.4:2.1[16].

TH isoform-specific, anti-oligopeptide antibodies were produced, and all four isoform proteins were detected in the human adrenal medulla and human

brain[17]. The estimated ratio of isoforms in the human adrenal medulla was 40:40:10:7. Since hTH3 and hTH4 mRNA contents were higher in the adrenal medulla than in the brain, the ratio of hTH1–4 proteins is thought to be similar to the ratio of TH isoform mRNAs present.

TH isoforms are also found in monkeys[18,19]. Analyses of mRNA and/or genomic DNA of marmosets (New-world monkeys), crab-eating monkeys (*Macaca irus*), Japanese monkeys (*Macaca fuscata*, Old-world monkeys) and gorillas using PCR indicate that multiple types of TH corresponding to hTH1 and hTH2 are present, and that the isoforms corresponding to hTH3 and hTH4 are absent. Chimpanzee, orangutan and gibbon were also suggested to have types 1 and 2 from the genomic DNA sequences, but these higher apes, except gorilla, may also have the capacity to produce type-3 and type-4 mRNA. Direct analysis of mRNAs would be required to determine the existence of types 3 and 4 in these anthropoids. Immunohistochemical studies have revealed that both type-1 and type-2 TH proteins, but not types 3 or 4, are expressed in the brain of macaque monkeys[20]. These results indicate that New- and Old-world monkeys and gorillas produce TH types 1 and 2, and that mutations that had accumulated in the genomic DNA create a new exon (exon 2 or exon 1_3), resulting in the appearance of two new TH isoforms, types 3 and 4, in humans[19]. Phylogenetic trees of hominoids suggest that the gibbon split off from the common ancestor first, followed by the orangutan and gorilla. Finally, the chimpanzee and human separated about 5 million years ago. Distances between gorilla and human, and between chimpanzee and human, are very close. The increased heterogeneity of TH, from a single isoform in non-primate animals, to two isoforms in monkeys and four isoforms in humans, offers new insight into the sequence of events leading to the evolution into separate species of the high primates.

Generation of heterogeneity in the TH isoforms in monkeys and humans may alter the biosynthesis of catecholamines *in vivo*, and might affect the growth of neurites and the neural circuitry in the brain. Since TH regulates the biosynthesis of catecholamines that are essential for higher brain function, it is tempting to speculate that the genetic difference among humans, primates and non-primates is related to a specific brain function.

Regulation of TH

TH is regulated in a very complex way: in the short term, TH activity is mainly regulated by phosphorylation of serine residues in the regulatory domain at the N-terminus by various protein kinases; in the long term, such as under stress, TH is regulated at the transcriptional level resulting in the induction of TH (Figure 9).

TH purified from various species is a 240 kDa homotetramer composed of four subunits of approx. 60 kDa each. Each of the purified human TH types 1–4 expressed in *E. coli* also has a tetrameric structure. Limited proteolysis

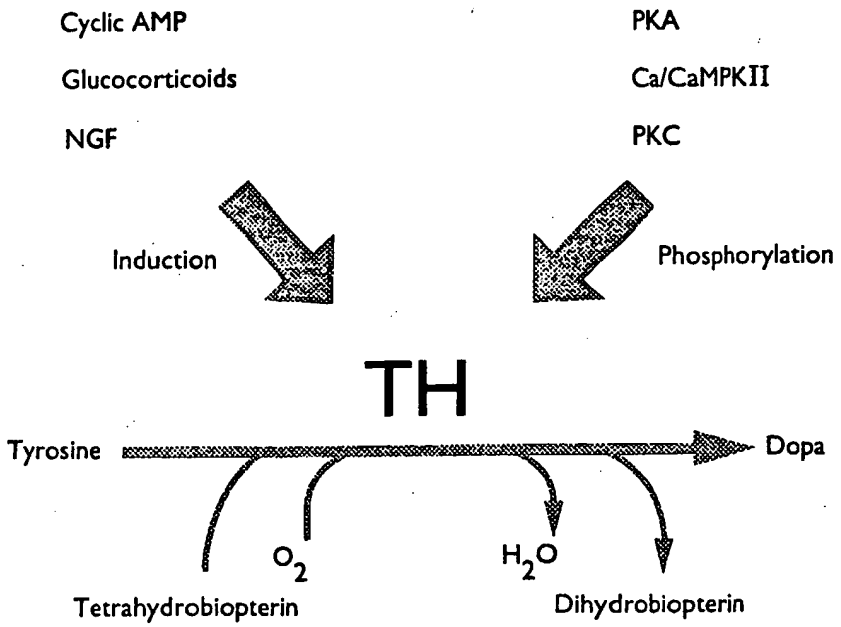


Figure 9. The mechanism of short-term regulation of TH by phosphorylation (activation) and long-term regulation by induction (gene expression)
Protein kinases (PKA, Ca/CaMPKII and PKC) activate TH via phosphorylation in the short term and also induce TH via gene activation in the long term.

reveals the inhibitory regulatory domain at the N-terminus and the catalytic domain at the C-terminus. Deletion mutagenesis studies place the C-terminal catalytic domain of rat TH between residues 158 and 184, and the carboxyl end at or prior to position 455[21]. Mature TH purified from adrenals or brain, or recombinant TH expressed in *E. coli*, exists as a homotetramer. A region containing a putative C-terminal leucine zipper may be required for TH tetramer formation[22].

Figure 10 shows a schematic presentation of the short-term regulation of dopamine biosynthesis via regulation of TH activity in the dopaminergic nerve terminals in the basal ganglia of the brain. The concentration of the cofactor, tetrahydrobiopterin, is a regulatory factor. TH is not saturated with tetrahydrobiopterin *in vivo*, and the cofactor level which is mainly regulated by GTP cyclohydrolase I activity may also regulate TH activity.

Catecholamines, the end product of the TH reaction, inhibit the enzyme activity competitively with tetrahydrobiopterin[1], and inactivate the enzyme reversibly to convert the active/labile form to an inactive/stable form[23]. These two feedback inhibition mechanisms by catecholamines are important in short-term regulation. Bovine adrenal TH is isolated in the inhibited state with catecholamines as the blue-green coloured catecholamine–Fe^{2+} complex. Phosphorylation of Ser-40 at pH 7.0 causes the release of catecholamine to activate the enzyme[24].

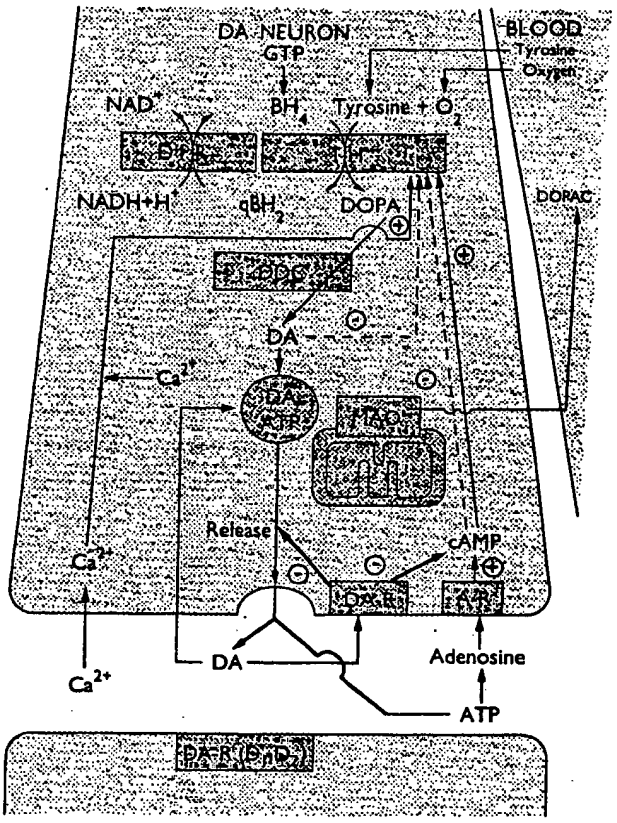


Figure 10. Schematic presentation of the short-term regulation of dopamine biosynthesis via TH activity in the dopaminergic nerve terminals in the basal ganglia of the brain

Abbreviations used: A-R, adenosine receptor; BH_4, tetrahydrobiopterin; cAMP, cyclic AMP; DA, dopamine; DA-R, dopamine receptor; DDC, dopa decarboxylase (AADC, aromatic L-amino acid decarboxylase); DOPA, L-dopa; DOPAC, 3,4-dihydroxyphenylacetic acid; DPR, dihydropteridine reductase; MAO, monoamine oxidase; qBH_2, quinonoid dihydrobiopterin.

Another probable regulation of TH activity is activation by association with chromaffin granules in the adrenal medulla or with synaptic vesicles. TH from the cytosol can bind reversibly to the granule membrane in a process that results in activation. This attachment of TH on the surface of chromaffin granules has been confirmed by immuno-electronmicroscopy[25].

The most important short-term mechanism for regulation of TH is activation by phosphorylation via protein kinases and deactivation by dephosphorylation via protein phosphatases. As shown in the schematic diagram of Figure 11, the main phosphorylation sites of TH in vitro are Ser-19, Ser-31 and Ser-40[26]. Ser-19 is phosphorylated mainly by Ca^{2+}/calmodulin-dependent protein kinase II (Ca/CaMPKII; EC 2.7.1.123), while Ser-40 is phosphorylated mainly by protein kinase A (PKA). Ca/CaMPKII may phosphorylate and activate TH of PC12h cells when they are depolarized by high K^+ because

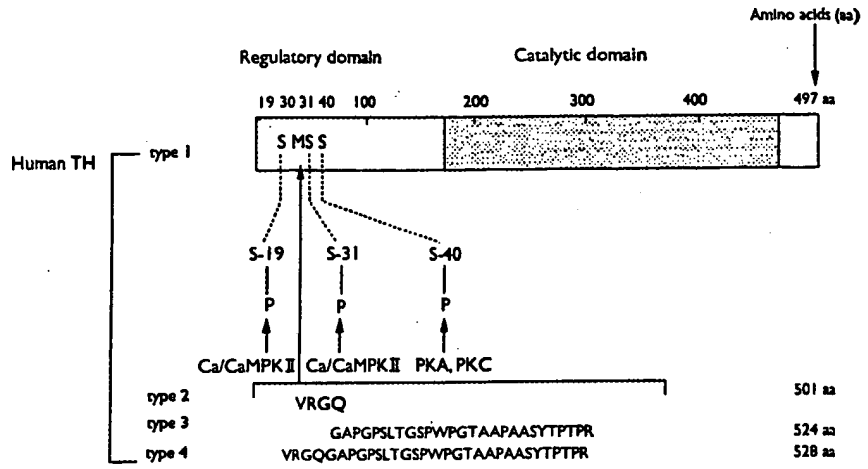


Figure 11. Schematic diagram of the main phosphorylation sites (Ser-19, Ser-31 and Ser-40) of hTH1

The open area shows the regulatory N-terminal domain. The shaded area shows the catalytic C-terminal domain. S-19, S-31 and S-40 are the main phosphorylation sites activating the enzyme. The insertion sequences of 4, 27 and 31 amino acids between M-30 and S-31 correspond to hTH2, hTH3 and hTH4.

a selective inhibitor of Ca/CaMPKII, KN-62 [1-[*N*,*O*-bis(5-isoquinolinsulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine], inhibits this TH phosphorylation and reduces dopamine synthesis. These results agree with the report that Ca/CaMPKII mediates phosphorylation of TH by hormonal and electrical stimuli, which leads to elevation of Ca^{2+} levels in PC12 cells[27]. Ser-40 is phosphorylated by PKA, protein kinase C (PKC) and Ca/CaMPKII; however, PKA phosphorylates Ser-40 of all four subunits of the enzyme molecule, causing a marked activation, whereas PKC and Ca/CaMPKII phosphorylate only two of the four subunits without affecting the enzyme activity[28]. Ser-31 is also phosphorylated by the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), two microtubule-associated protein kinases[29].

The first messengers likely to regulate TH phosphorylation include: dopamine (via the presynaptic dopamine autoreceptor); adenosine (via the presynaptic A2 receptor); glutamate [via the *N*-methyl-D-aspartate (NMDA) receptor]; vasoactive intestinal polypeptide (VIP) (via PKA); angiotensin (via PKC); secretin–glucagon (via PKA); prolactin (via PKC); and nerve growth factor (NGF) (via Ca/CaMPKII). Dephosphorylation of TH by protein phosphatases (type 2A) decreases the activity. However, the enzyme expressed in *E. coli* has high activity without phosphorylation, indicating that the unphosphorylated enzyme has activity. Another finding, suggesting the regulatory inter-relationship between the tetrahydrobiopterin synthetic pathway and catecholamine biosynthesis, is that tetrahydrobiopterin activates TH phosphatase.

Thus increased concentrations of tetrahydrobiopterin activate TH, but may also decrease its activity due to dephosphorylation[30].

The mechanism of activation of TH by phosphorylation at Ser-40 is increased affinity for the tetrahydrobiopterin cofactor and removal of inhibition by the end-product catecholamine[31]. The insertion sequence between Met-30 and Ser-31 of hTH1 promotes additional phosphorylation of hTH2 by Ca/CaMPKII. Unlike hTH1, phosphorylation of hTH2 by Ca/CaMPKII results in an increase of the K_i value for dopamine, giving a greater potential for activation than hTH1. The hTH1–4 isoforms are phosphorylated at Ser-40 and Ser-19 by mitogen-activated protein-kinase (MAP kinase)-activated kinase-1 and -2 (MAPKAP kinase-1 and -2), and at Ser-31 by MAP kinase. It is suggested that phosphorylation by MAPKAP kinase-1 and -2 may be of particular importance for the regulation of hTH2, which is phosphorylated by MAP kinase very poorly, and that phosphorylation by MAP kinase may be of special significance for the regulation of hTH3 and hTH4[32].

hTH1, hTH2 and hTH4 are inhibited by catecholamines in competition with tetrahydrobiopterin. Catecholamines bind to hTH1 and hTH2 with a stoicheiometry of about 1 mol per mol of enzyme subunit interacting with the catalytic iron at the active site. Tetrahydrobiopterin causes a dissociation of dopamine from hTH1. Phosphorylation at Ser-40 by PKA decreases the affinity of dopamine binding by a factor of 10. These results suggest that human TH isoforms are regulated in a similar fashion to TH from other species.

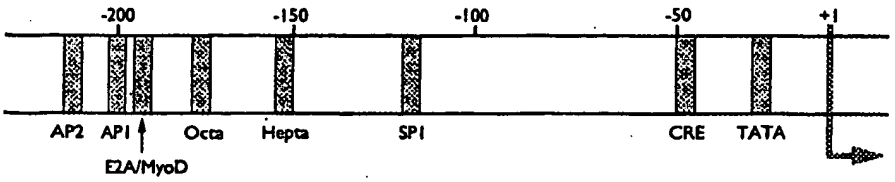
Gene expression of TH

TH is regulated in the long term, such as under chronic stress, by enzyme induction at the transcriptional level. As shown in Figure 12, several putative regulatory elements exist in the 5′-upstream region of the genes of human and rat TH within 0.2 kb of the 5′-flanking DNA sequence: AP2, AP1, POU/Octa, Hepta, Sp1 and cyclic AMP response element (CRE).

Protein kinases (PKA, Ca/CaMPKII, PKC etc.) activate TH by phosphorylation in the short term, and also induce TH protein in the long term. Thus protein kinases have dual regulatory roles.

The expression of TH in cultured cells and tissues containing catecholamines is regulated by various first messengers (e.g. dopamine, dopamine

Figure 12. A schematic map of the 5′ upstream region of the human TH gene



agonists and antagonists, dexamethasone, VIP and secretin, angiotensin II, bradykinin, neurotensin and NGF), and by PKA and PKC signal transduction pathways.

Functional CRE activity has been found in a variety of cell lines. The CRE appears to play an important dual role: as a basal promoter element and an inducible enhancer for TH transcription. CRE and CRE-binding protein (CREB) may play a fundamental role in the transcriptional activation of the TH gene in catecholaminergic cells[33,34].

The AP1 site may also functionally regulate TH gene activity, but may contribute to transcription to a smaller degree than CRE. Binding of the products of immediate early genes, c-Fos- and c-Jun-related proteins induced by NGF or angiotensin II, to the putative AP1-like sites increases TH transcription. Thus NGF treatment of responsive cells increases TH action by two different mechanisms: the first is a short-term elevation of TH activity due to an increase in TH phosphorylation; the second is a long-term elevation of TH due to an increase in the synthesis of the enzyme. Both PKA and PKC appear both to activate TH by phosphorylation and to induce its synthesis by an increase in TH transcription. It has also been proposed that the tissue-specific regulation of TH requires a synergistic interaction between the AP1 motif and the overlapping E-box.

Cold-induced increases in adrenomedullary TH gene expression are mediated through the interaction of the AP1 binding site and the c-Fos/c-Jun. Membrane depolarization induces an increase in intracellular Ca^{2+} which in turn induces TH. The depolarization response element in the TH gene in PC12 cells is thought to be CRE. Thus CRE appears to be functioning as a calcium regulatory element in this system.

Analysis of TH gene expression in transgenic mice

Since the expression pattern of TH is spatially and temporally specific, transgenic mice are useful for defining the regulation elements for TH gene expression. The transgenic (HTH) mice carrying an 11 kb fragment (containing a 2.5 kb 5′-flanking region, the entire exon–intron sequence and a 0.5 kb 3′-flanking region) exhibited high-level and tissue-specific expression of human TH in the brain and adrenal glands[35]. The 5.0 kb 5′-flanking region of the human TH gene could drive chloramphenicol acetyltransferase (CAT) reporter gene expression in catecholaminergic neurons and adrenal medullary cells of non-transgenic mice; however, CAT expression was also observed in some non-catecholaminergic neurons, including those in several sites where transient TH expression has been reported. The 2.5 kb and 0.2 kb 5′-flanking fragments of the TH gene could not express CAT in catecholaminergic neurons[36]. The 5.0 kb of the human TH 5′-flanking region, the exon–intron structure and/or 3′-flanking region of the TH gene may function in catecholaminergic neuron-specific expression. The results in HTH transgenic mice

show that the fundamental cellular machinery necessary for the alternative splicing of human TH mRNA is present and functioning in the mouse catecholaminergic cells and produces multiple forms of the enzyme from human TH mRNA sequence. Although human TH mRNA and active protein are overexpressed in the HTH transgenic mice, the catecholamine levels and phenotypes are not significantly different from those of non-transgenic mice, suggesting that there are other, unknown, regulatory mechanisms for the catecholamine levels in the transgenic mice. In transgenic mice, introducing either 4.8 kb or 9 kb of the 5′ flanking region of the rat TH gene is sufficient for the high level of tissue-specific expression[37,38]. Thus it may also be possible that other catecholaminergic neuron-specific elements reside between 5 kb and 9 kb of the human TH gene, as well as in the intron–exon structure and/or the 3′-terminal region.

TH in disease

Since catecholamines are closely related to the pathogenesis of neuropsychiatric or cardiovascular disorders, TH has been suggested to play an important role in a number of diseases.

In Parkinson's disease, TH activity, protein levels and mRNA levels are decreased in the nigrostriatal dopaminergic neurons[39]. A quantitative RT-PCR method for the four types of human TH mRNA revealed that the four isoforms exist in the human substantia nigra at an approximate ratio of 45:50:2:3. In Parkinsonian substantia nigra, each form of TH mRNA was decreased to about 25% of the normal level (Figure 13). In contrast, neither the absolute amount nor the ratio of hTH1–4 protein changed in schizophrenia. If hTH1–4 mRNAs exist in the same neuron, the total amount but not the ratio of hTH1–4 may change. On the other hand, neurons containing only one type

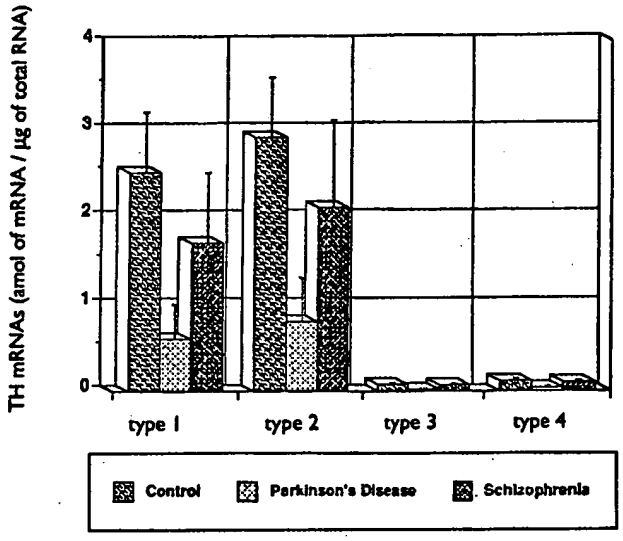


Figure 13. Quantification of mRNAs of human TH isoforms in the substantia nigra[16] Total amounts of TH mRNA (amol TH mRNA/μg of total RNA) in control, Parkinson's disease and schizophrenia were 5.4 ± 1.4, 1.5 ± 0.9 and 4.0 ± 1.8, respectively. The total amount, type 1 and 2 mRNAs were significantly reduced compared with corresponding control values ($P<0.05$).

of human TH protein were demonstrated by immunohistochemistry[17]. It is interesting that the surviving dopaminergic neurons in Parkinson's disease have decreased levels of TH mRNA and protein, suggesting the incapability of the remaining neurons[40].

The nigrostriatal dopamine neurons appear to be the most susceptible to dopamine deficiency. The first symptom (phenotype) of dopamine deficiency may be dystonia, i.e. disordered tonicity of muscle. Hereditary progressive dystonia with marked diurnal fluctuation (HPD) (also called dopa-responsive dystonia, DRD) is a dystonia with autosomal dominant inheritance with dopamine deficiency in the nigro-striatum of the brain, originally described by Segawa and sometimes known as Segawa's syndrome. Small doses of L-dopa can cure the patients. HPD/DRD is caused by mutation of GTP cyclohydrolase I, the first enzyme in tetrahydrobiopterin biosynthesis. The resultant decrease in tetrahydrobiopterin, to below 20% of the normal level, causes the decreased TH activity and dopamine deficiency[41]. A recessive inherited form of HPD/DRD in other families is caused by point mutation of TH (Gln-381 → Lys)[42]. It should be noted that another autosomal recessive condition of GTP cyclohydrolase I deficiency is also caused by a point mutation, resulting in no enzyme activity and severe neurological symptoms[43].

Catecholamine neurotransmitters are assumed to be closely related to mental diseases, especially to bipolar affective disorders (manic depressive illness) or schizophrenia. The TH gene is a part of a gene cluster of TH–insulin gene–insulin-like growth factor 2 gene in human chromosome 11p15.5. The TH gene is 5′ to the insulin gene and is separated by only 2.7 kb of flanking DNA. Since the first report in 1981 on linkage study, suggesting association between TH and the bipolar affective disorders, extensive studies have been carried out with conflicting results. A positive association between the bipolar illness and a locus containing the gene for TH has been reported[44]. Increased TH activity is assumed to explain probable overactivity of ventro-tegmental dopaminergic neurons in schizophrenia, but the amounts of hTH1–4 mRNAs in the substantia nigra do not change from those from normal controls[16]. It is obviously necessary to carry out molecular genetic studies with increased numbers of patients and families with affective disorders or schizophrenia.

An interesting therapeutic approach for Parkinson's disease is brain transplantation of non-neuronal cells transfected with human TH gene. This work is still at the level of animal experimentation. One important factor is the availability of the cofactor of TH, tetrahydrobiopterin[45], since tetrahydrobiopterin is essential for TH activity. Use of an adenovirus vector for gene transfer of TH into the substantia nigra by stereotaxic inoculation may be a promising approach as a gene therapy for Parkinson's disease.

Summary

- *TH is a tetrahydrobiopterin-requiring, iron-containing monooxygenase. It catalyses the conversion of L-tyrosine to L-dopa, which is the first, rate-limiting step in the biosynthesis of catecholamines (dopamine, noradrenaline and adrenaline), the central and sympathetic neurotransmitters and adrenomedullary hormones. The cofactor of TH is tetrahydrobiopterin, which is synthesized from GTP in three steps.*
- *The TH gene consists of 14 exons only in humans and 13 exons in animals. Human TH exists in four isoforms (hTH1–4) that are produced by alternative mRNA splicing from a single gene. A single mRNA and protein corresponding to hTH1 exists in non-primates. Monkey TH exists in two isoforms, corresponding to hTH1 and hTH2.*
- *TH activity is regulated in the short term by feedback inhibition of catecholamines in competition with tetrahydrobiopterin, and by activation and deactivation due to phosphorylation and dephosphorylation, mainly at Ser-19 and Ser-40 of hTH1. The multiple TH isoforms in humans and monkeys have additional phosphorylation, resulting in more subtle regulation.*
- *In long-term regulation under stress conditions, TH protein is induced. CRE and AP1 in the 5′ flanking region of the TH gene may be the main functional elements for TH gene expression.*
- *TH may be closely related to the pathogenesis of neurological diseases, such as dystonia and Parkinson's disease, psychiatric diseases, such as affective disorders and schizophrenia, as well as cardiovascular diseases.*
- *The TH gene may prove useful in gene therapy to compensate for decreased levels of catecholamines in neurological diseases, for example, for supplementation of dopamine in Parkinson's disease.*

I apologize to the many contributors to the field to whom I have not been able to refer, owing to the limitation of reference numbers. I thank the Ministry of Education, Science and Culture of Japan, and Fujita Health University for their support.

Further reading

Nagatsu, T. (1991) Genes for human catecholamine-synthesizing enzymes. *Neurosci. Res.* **12**, 315–345

Naoi, M. & Parvez, S.H., eds. (1993) *Tyrosine Hydroxylase*, VSP, Utrecht

Kaufman, S., ed. (1985) Metabolism of aromatic amino acids and amines. *Methods Enzymol.* **142**, Academic Press, New York

Zigmond, R.E., Schwartzschild, M.A. & Rittenheuse, A.R. (1989) Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. *Annu. Rev. Neurosci.* **12**, 415–461

References

1. Nagatsu, T., Levitt, M. & Udenfriend, S. (1964) Tyrosine hydroxylase: the initial step in norepinephrine biosynthesis. *J. Biol. Chem.* **239**, 2910–2917
2. Levitt, M., Spector, S., Sjoerdsma, A. & Udenfriend, S. (1965) Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharmacol. Exp. Therap.* **148**, 1–8
3. Brenneman, A.R. & Kaufman, S. (1964) The role of tetrahydropteridines in the enzymatic conversion of tyrosine to 3,4-dihydroxyphenylalanine. *Biochem. Biophys. Res. Commun.* **17**, 177–183
4. Lazarus, R., Benkovic, S. & Kaufman, S. (1983) Phenylalanine hydroxylase stimulator protein is a 4a-carbinolamine dehydratase. *J. Biol. Chem.* **238**, 10960–10962
5. Nagatsu, T. & Oka, K. (1987) Tyrosine 3-monooxygenase from bovine adrenal medulla. *Methods Enzymol.* **142**, 56–62
6. Fujisawa, H. & Okuno, S. (1987) Tyrosine 3-monooxgenase from rat adrenals. *Methods Enzymol.* **142**, 63–71
7. Tank, A.W. & Weiner, N. (1987) Tyrosine 3-monooxygenase from rat pheochromocytoma. *Methods Enzymol.* **142**, 71–82
8. Mogi, M., Kojima, K. & Nagatsu, T. (1984) Detection of inactive or less active forms of tyrosine hydroxylase in human brain and adrenal by a sandwich enzyme immunoassay. *Anal. Biochem.* **138**, 125–132
9. Grima, B., Lamouroux, A., Blanot, F., Biguet, N.F. & Mallet, J. (1985) Complete coding sequence of rat tyrosine hydroxylase mRNA. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 617–621
10. Grima, B., Lamouroux, A., Boni, C., Julien, J.-F., Javoy-Agid, F. & Mallet, J. (1987) A single human gene encoding multiple tyrosine hydroxylases with different predicted functional characteristics. *Nature (London)* **326**, 707–711
11. Kaneda, N., Kobayashi, K., Ichinose, H., *et al.* (1987) Isolation of a novel cDNA clone for human tyrosine hydroxylase: alternative RNA splicing produces four kinds of mRNA from a single gene. *Biochem. Biophys. Res. Commun.* **146**, 971–975
12. O'Malley, K.L., Anhalt, M.J., Martin, B.M., Kalsoe, J.R., Winfield, S.L. & Ginns, E.I. (1987) Isolation and characterization of the human tyrosine hydroxylase gene: identification of 5' alternative splice sites responsible for multiple mRNAs. *Biochemistry* **26**, 6910–6914
13. Kobayashi, K., Kaneda, N., Ichinose, H., *et al.* (1988) Structures of the human tyrosine hydroxylase gene: alternative splicing from a single gene accounts for generation of four mRNA subtypes. *J. Biochem.* **103**, 907–912
14. Le Bourdellès, B., Horellou, P., Le Caer, J.-P., *et al.* (1991) Phosphorylation of human recombinant tyrosine hydroxylase isoforms 1 and 2: an additional phosphorylated residue in isoform 2, generated through alternative splicing. *J. Biol. Chem.* **266**, 17124–17130
15. Nasrin, S., Ichinose, H., Hidaka, H. & Nagatsu, T. (1994) Recombinant human tyrosine hydroxylase types 1–4 show regulatory kinetic properties for the natural (6R)-tetrahydrobiopterin cofactor. *J. Biochem.* **116**, 393–398
16. Ichinose, H., Ohye, T., Fujita, K., *et al.* (1994) Quantification of mRNA of tyrosine hydroxylase and aromatic L-amino acid decarboxylase in the substantia nigra in Parkinson's disease and schizophrenia. *J. Neural Transm. [P-D Sect]* **8**, 149–158
17. Lewis, D.A., Melchitzky, D.S. & Haycock, J.W. (1993) Four isoforms of tyrosine hydroxylase are expressed in human brain. *Neuroscience* **54**, 477–492

18. Ichikawa, S., Ichihose, H. & Nagatsu, T. (1990) Multiple mRNAs of monkey tyrosine hydroxylase. *Biochem. Biophys. Res. Commun.* **173**, 1331–1336
19. Ichinose, H., Ohye, T., Fujita, K., Yoshida, M., Ueda, S. & Nagatsu, T. (1993) Increased heterogeneity of tyrosine hydroxylase in humans. *Biochem. Biophys. Res. Commun.* **195**, 158–165
20. Lewis, D.A. Melchitzky, D.S. & Haycock, J.W. (1994) Expression and distribution of two isoforms of tyrosine hydroxylase in macaque monkey brain. *Brain Res.* **656**, 1–13
21. Ribeiro, P., Wang, Y., Citron, B.A. & Kaufman, S. (1993) Deletion mutagenesis of rat PC12 tyrosine hydroxylase regulatory and catalytic domains. *J. Mol. Neurosci.* **4**, 125–139
22. Vrana, K.E., Walker, S.J., Rucker, P. & Liu, X. (1994) A carboxyterminal leucine zipper is required for tyrosine hydroxylase tetramer formation. *J. Neurochem.* **63**, 2014–2020
23. Okuno, S. & Fujisawa, H. (1991) Conversion of tyrosine hydroxylase to stable and inactive form by the end product. *J. Neurochem* **57**, 53–60
24. Andersson, K.K., Cox, D.D., Que, L., Jr., Flatmark, T. & Haavik, J. (1988) Resonance raman studies on the blue-green-coloured bovine adrenal tyrosine 3-monooxygenase (tyrosine hydroxylase). Evidence that the feedback inhibitors adrenaline and noradrenaline are coordinated to iron. *J. Biol. Chem.* **263**, 1821–18626
25. Nagatsu, I., Karasawa, N., Kondo, Y. & Inagaki, S. (1979) Immunocytochemical localization of tyrosine hydroxylase, dopamine-β-hydroxylase and phenylethanolamine-*N*-methyltransferase in the peroxidase-antiperoxidase method. *Histochemistry* **64**, 131–144
26. Campbell, D.G., Hardie,D.G. & Vulliet, P.R. (1986) Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. *J. Biol. Chem.* **261**, 10489–10492
27. Ishii, A., Kiuchi, K.,Kobayashi, R., Sumi, M., Hidaka, H. & Nagatsu, T. (1991) A selective Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, KN-62, inhibits the enhanced phosphorylation and the activity of tyrosine hydroxylase by 56 mM K^+ in rat pheochromocytoma PC12h cells. *Biochem. Biophys. Res. Commun* **176**, 1051–1056
28. Funakoshi, H., Okuno, S. & Fujisawa, H. (1991) Different effects on activity caused by phosphorylation of tyrosine hydroxylase at serine 40 by three multifunctional protein kinases. *J. Biol. Chem.* **266**, 15614–15620
29. Haycock, J.W., Ahn, N.G., Cobb, M.H. & Krebs, E.G. (1992) ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 *in situ*. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2365–2369
30. Nelson, T.J. & Kaufman, S. (1987) Activation of rat caudate tyrosine hydroxylase phosphatase by tetrahydrobiopterin. *J. Biol. Chem.* **262**, 16470–16475
31. Bailey, S.W., Dillard, S.B., Thomas, K.B. & Ayling, J.E. (1987) Changes in the cofactor binding domain of bovine striatal tyrosine hydroxylase at physiological pH upon cAMP-dependent phosphorylation mapped with tetrahydrobiopterin analogues. *Biochemistry* **28**, 494–504
32. Sutherland, D., Alterio, J., Campbell, D.G., *et al.* (1993) Phosphorylation and activation of human tyrosine hydroxylase *in vitro* by mitogen-activated protein (MAP) kinase and MAP-kinase activated kinases 1 and 2. *Eur. J. Biochem.* **217**, 715–722
33. Cambi, F., Fung, B. & Chikaraishi, D. (1989) 5′ Flanking DNA sequences direct cell-specific expression of rat tyrosine hydroxylase. *J. Neurochem.* **53**, 1656–1659
34. Kim, K.-T., Park, D.H. & Joh, T.H. (1993) Parallel up-regulation of catecholamine biosynthetic enzymes by dexamethasone in PC12 cells. *J. Neurochem.* **60**, 946–951
35. Kaneda, N., Sasaoka, T., Kobayashi, K., *et al.* (1991) Tissue-specific and high-level expression of the human tyrosine hydroxylase gene in transgenic mice. *Neuron* **6**, 583–594
36. Sasaoka T., Kobayashi, K., Nagatsu, I., *et al.* (1992) Analysis of the human tyrosine hydroxylase promoter-chloramphenicol acetyltransferase chimeric gene expression in transgenic mice. *Mol. Brain. Res.***16**, 274–286
37. Banerjee, S.A., Roffler-Tarlov, S., Szabo, M., Frohman, L. & Chikaraishi, D.M. (1994) DNA regulatory sequences of the rat tyrosine hydroxylase gene direct correct catecholaminergic cell-type specificity of a human growth hormone receptor in the CNS of transgenic mice causing a dwarf phenotype. *Mol. Brain Res.* **24**, 89–106.

38. Min, N., Joh, T.H., Kim, K.S., Peng, C. & Son, J.H. (1994) 5′ Upstream DNA sequence of the rat tyrosine hydroxylase gene directs high-level and tissue-specific expression to catecholaminergic neurons in the central nervous system of transgenic mice. *Mol. Brain Res.* **27**, 281–289
39. Nagatsu, T. (1990) Change of tyrosine hydroxylase in the parkinsonian brain and in the brain of MPTP-treated mice as revealed by homospecific activity. *Neurochem. Res.* **15**, 425–429
40. Kastner, A., Hirsch, E.C., Agid, Y. & Javoy-Agid, F. (1993) Tyrosine hydroxylase and messenger RNA in the dopaminergic nigral neurons of patients with Parkinson's disease. *Brain Res.* **606**, 341–345
41. Ichinose, H., Ohye, T., Takahashi, T., *et al.* (1994) Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene. *Nature Genet.* **8**, 236–242
42. Lüdecke, B., Dworniezak, B. & Bartholomé, K. (1995) A point mutation in the tyrosine hydroxylase gene associated with Segawa's Syndrome. *Human Genet.* **95**, 123–125
43. Blau, N., Ichinose, H., Nagatsu, T., Heizmann, C.W., Zacchello, F. & Burline, A.B. (1995) A missense mutation in a patient with GTP cyclohydrolase I deficiency missed in the newborn screening program. *J. Pediatr.*, **126**, 401–405
44. Leboyer, M., Malafosse, A., Boularands, S., *et al.* (1990) Tyrosine hydroxylase polymorphisms associated with manic-depressive illness. *Lancet* **335**, 1219
45. Uchida, K., Tsuzaki, N., Nagatsu, T. & Kohsaka, S. (1992) Tetrahydrobiopterin-dependent functional recovery in 6-hydroxydopamine-treated rats by intracerebral grafting of fibroblasts transfected with tyrosine hydroxylase cDNA. *Devel. Neurosci.* **14**, 173–180

EXHIBIT O

United States Patent [19]

Naruse et al.

[11] Patent Number: 4,778,794

[45] Date of Patent: Oct. 18, 1988

[54] PHARMACEUTICAL COMPOSITION FOR THE TREATMENT OF INFANTILE AUTISM

[75] Inventors: Hiroshi Naruse, Tokyo; Masashi Takesada, Hyogo; Osamu Hayaishi; Yasuyoshi Watanabe, both of Kyoto, all of Japan

[73] Assignee: Suntory Limited, Osaka, Japan

[21] Appl. No.: 870,495

[22] Filed: Jun. 4, 1986

[30] Foreign Application Priority Data

Jun. 4, 1985 [JP] Japan 60-121347

[51] Int. Cl.[4] A61K 31/50; A61K 31/495

[52] U.S. Cl. 514/254

[58] Field of Search 514/254

[56] References Cited

U.S. PATENT DOCUMENTS

4,665,182 5/1987 Nichol et al. 544/258

4,701,455 10/1987 Nichol et al. 544/258

Primary Examiner—Stanley J. Friedman

Attorney, Agent, or Firm—Cushman, Darby & Cushman

[57] ABSTRACT

A pharmaceutical composition for the treatment of infantile autism which contains tetrahydrobiopterin or a derivative thereof as a major effective ingredient and 5-hydroxytryptophan and/or L-DOPA as an optional auxiliary effective ingredient is provided.

5 Claims, 5 Drawing Sheets

Fig. 1

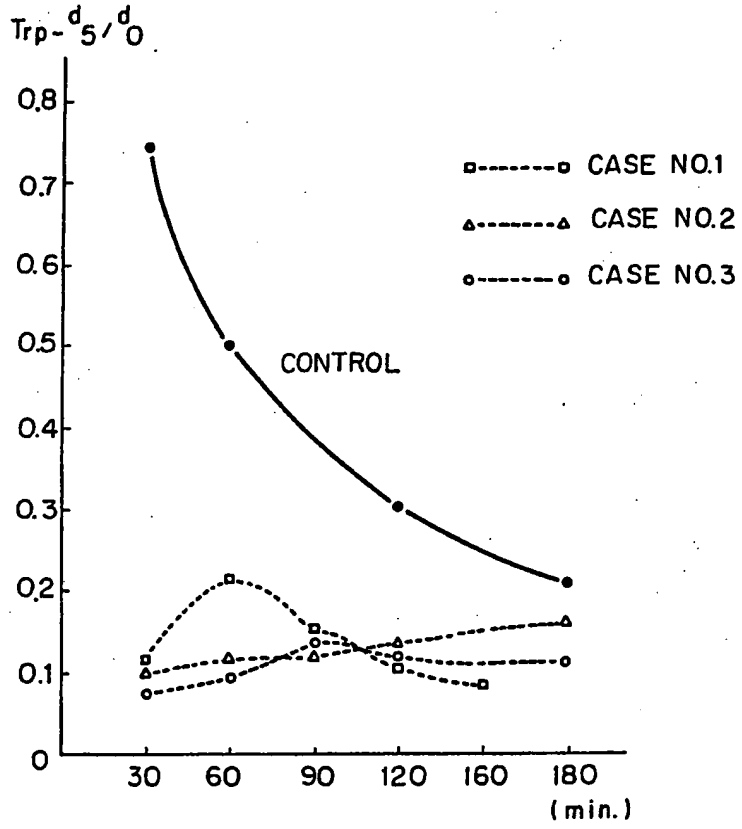

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Fig. 2

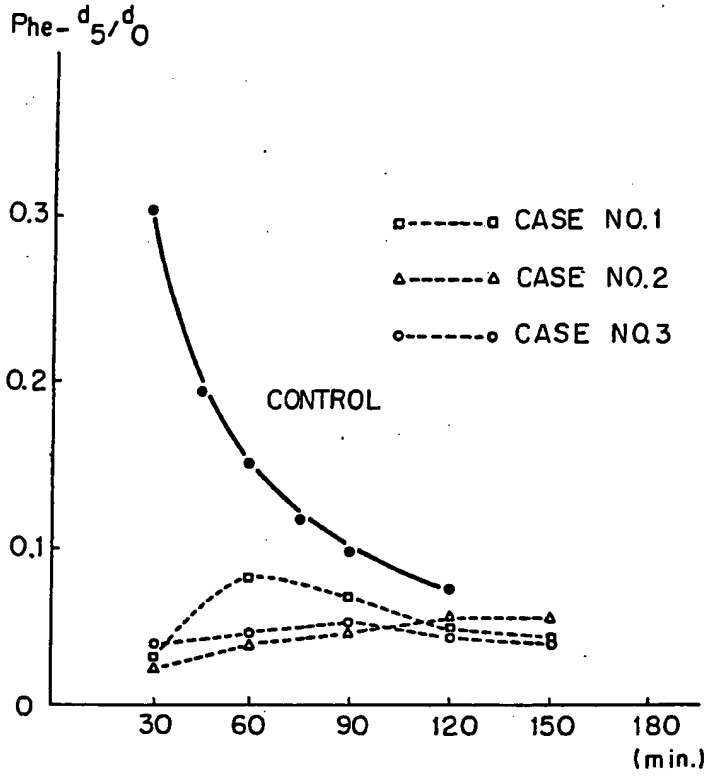
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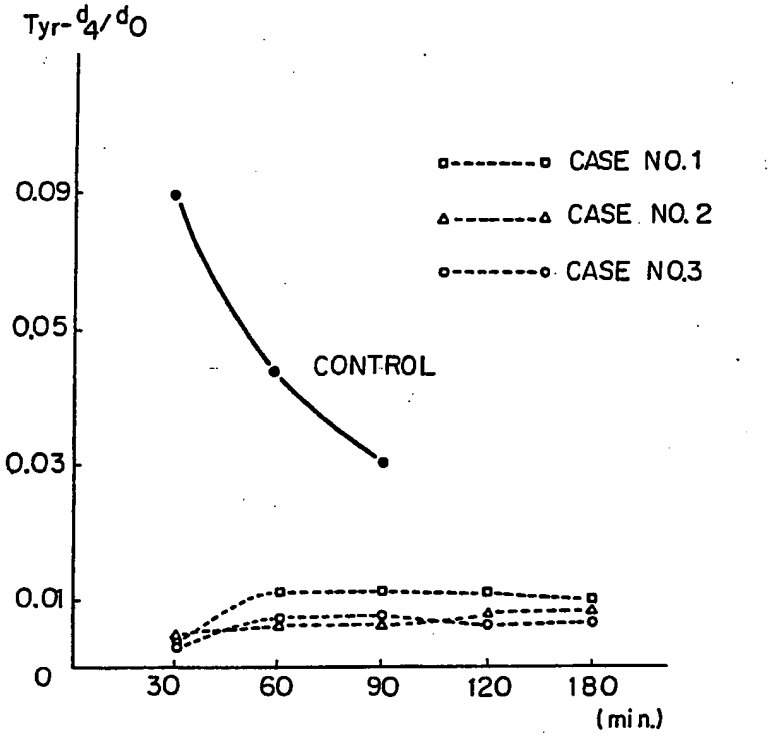
Fig. 3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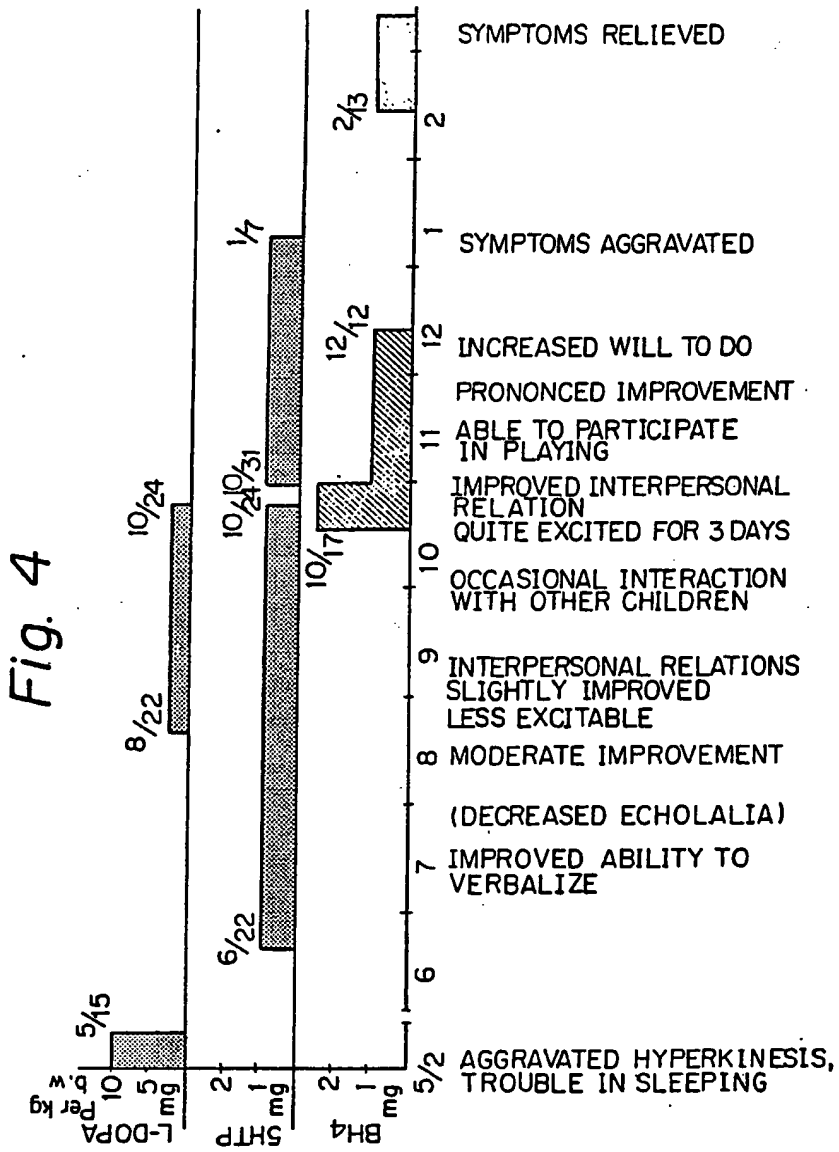
SYMPTOMS RELIEVED
SYMPTOMS AGGRAVATED
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PRONONCED IMPROVEMENT
ABLE TO PARTICIPATE IN PLAYING
IMPROVED INTERPERSONAL RELATION
QUITE EXCITED FOR 3 DAYS
OCCASIONAL INTERACTION WITH OTHER CHILDREN
INTERPERSONAL RELATIONS SLIGHTLY IMPROVED
LESS EXCITABLE
MODERATE IMPROVEMENT
(DECREASED ECHOLALIA)
IMPROVED ABILITY TO VERBALIZE
AGGRAVATED HYPERKINESIS, TROUBLE IN SLEEPING
L-DOPA
5HTP
BH4
mg
Per kg b.w.
2/13
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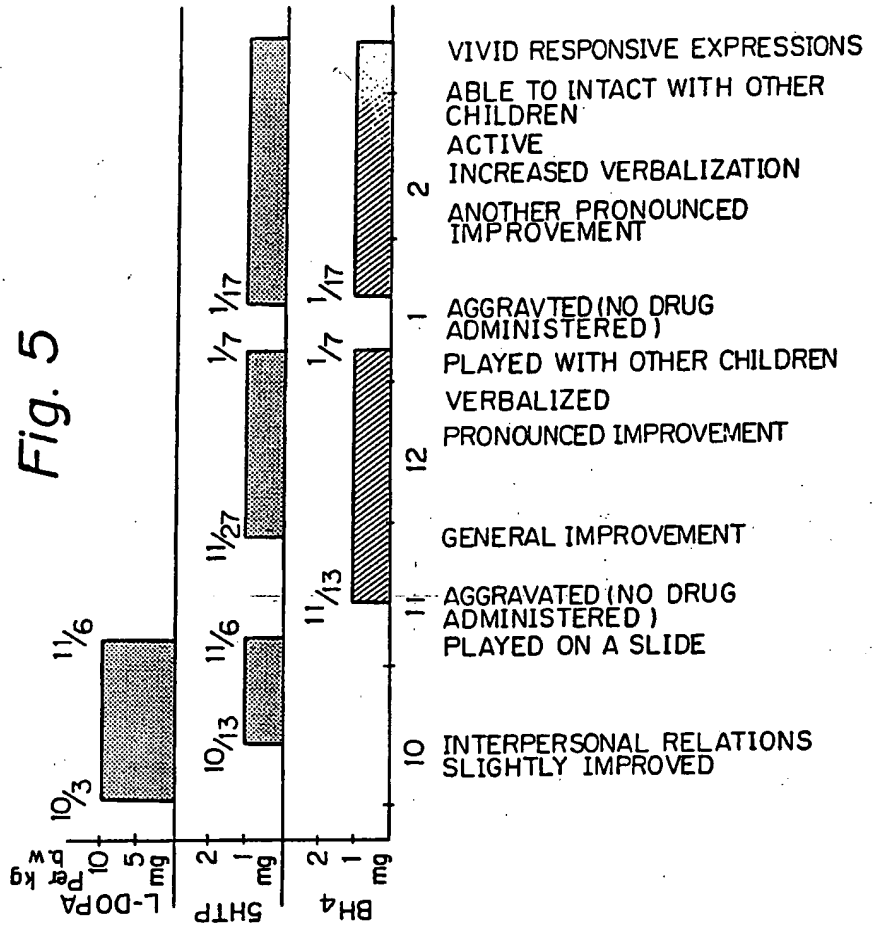
5/2

Fig. 4

Fig. 5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12
11
10
VIVID RESPONSIVE EXPRESSIONS
ABLE TO INTACT WITH OTHER CHILDREN
ACTIVE
INCREASED VERBALIZATION
ANOTHER PRONOUNCED IMPROVEMENT
AGGRAVTED (NO DRUG ADMINISTERED)
PLAYED WITH OTHER CHILDREN
VERBALIZED
PRONOUNCED IMPROVEMENT
GENERAL IMPROVEMENT
AGGRAVATED (NO DRUG ADMINISTERED)
PLAYED ON A SLIDE
INTERPERSONAL RELATIONS SLIGHTLY IMPROVED

PHARMACEUTICAL COMPOSITION FOR THE TREATMENT OF INFANTILE AUTISM

FIELD OF THE INVENTION

The present invention relates to a pharmaceutical composition for the treatment of infantile autism which contains tetrahydrobiopterin or a derivative thereof as an effective ingredient.

PRIOR ART

Ever since the finding of the dysfunctioning of the brain in autistic children, autism has been considered to be a disease caused by brain impairment. The etiology of autism has been ascribed to heredity, developmental anomaly or impairment at delivery but no lucid and convincing explanation has yet been put forward. Therefore, the treatments so far tried have been limited to nosotropic ones which involve the administration of such drugs as pimozide, haloperidol, pentoxyfylline and calcium hopantenate in accordance with the specific abnormal behaviors manifested by autistic patients, and no treatment which is truly etiotropic has been known [Acta paedopsychiat., 48, 173–184 (1982); Clin. Eval., 8, 629–673, December, 1980; Shinryo to Shinyaku (Diagnosis and New Drugs), 21, 4, Special Issue, Apr. 1, 1984).

Tetrahydrobiopterin and derivatives thereof are known compounds which have been used in the treatment of malignant hyperphenylalaninemia, depressions and Parkinson's disease (see, for example, Japanese Patent Public Disclosure Nos. 25323/1984 and 76086/1984).

As mentioned above, however, no etiotropic drug for the treatment of autism has been found and there exists a strong need to develop such a drug.

SUMMARY OF THE INVENTION

The present inventors obtained observations that indicate impaired cellular transport of aromatic amino acids in representative cases of children suffering from infantile autism. They therefore postulated that insufficiency of serotonin and catecholamines in the brain could cause autism and on the basis of this hypothesis, the inventors administered 5HTP (5-hydroxytryptophan) and L-DOPA (i.e., precursors for serotonin and catecholamines) to autistic children. In many cases, their symptoms were generally relieved but, in some cases, the symptoms were aggravated, probably because of overdosing of these drugs.

Therefore, instead of administering these two precursors, the present inventors used tetrahydrobiopterin which is a coenzyme for the hydroxylase of aromatic amino acids and which is a rate-limiting factor for the synthesis of serotonin and catecholamines. This compound turned out be surprisingly effective in the treatment of autism. The present invention has been accomplished on the basis of this finding.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph depicting the blood level of Trp-d_5 as a function of time after it was administered per-orally to autistic children;

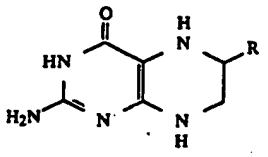
FIG. 2 is a graph showing the blood level of Phe-d_5 as a function of time after it was administered pre-orally to autistic children;

FIG. 3 is a graph showing the blood level of Tyr-d_4 as a function of time after it was administered pre-orally to autistic children; and

FIGS. 4 and 5 are charts which outline the time schedule of administration of BPH_4, 5HTP and L-DOPA and the resulting changes in the symptoms of Cases 1 and 2, respectively.

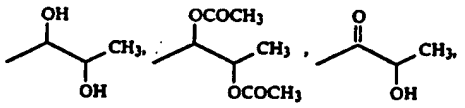
DETAILED DESCRIPTION OF THE INVENTION

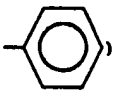
The present invention relates to a pharmaceutical composition for the treatment of autism which contains a compound of the formula (I):



(I)

(wherein R is

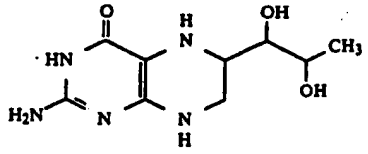


—CH_3, CH_2OH or — )

or a salt thereof as an effective ingredient.

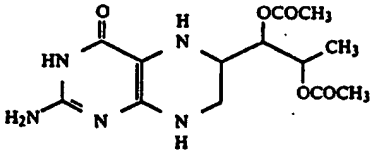
Examples of an effective ingredient in the pharmaceutical composition of the present invention include the compounds listed below and salts thereof:

(1) L-erythro-5,6,7,8-tetrahydrobiopterin:

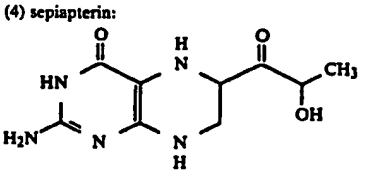


(2) DL-5,6,7,8-tetrahydrobiopterin:

(3) 1',2'-diacetyl-5,6,7,8-tetrahydrobiopterin:

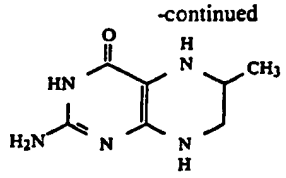


(4) sepiapterin:

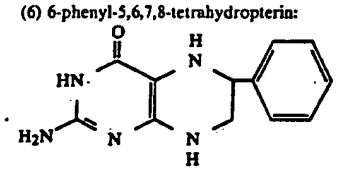


(5) 6-methyl-5,6,7,8-tetrahydropterin:

-continued



(6) 6-phenyl-5,6,7,8-tetrahydropterin:



Among the compounds shown above, 5,6,7,8-tetrahydrobiopterin and salts thereof are preferable. In consideration of several factors such as toxicity, L-erythro-5,6,7,8-tetrahydrobiopterin and salts thereof are most preferable.

The compounds of formula (I) are known and are described in, for example, Japanese Patent Public Disclosure Nos. 25323/1984 and 76086/1984. Illustrative salts are those with pharmaceutically nontoxic acids such as hydrochloric acid, phosphoric acid, sulfuric acid, boric acid, acetic acid and formic acid. Salts of the compounds of formula (I) with such acids are also included in the definition of the "active ingredient" in the pharmaceutical composition of the present invention.

The pharmaceutical compositions of the present invention may be prepared by formulating them in dosage forms which are suitable for peroral, rectal or nonparenteral administration, the last-mentioned including intravenous injection and administration into the cerebrospinal fluid. For this purpose, common carriers and routine formulation techniques may be employed.

"Common carriers" means those which are employed in standard pharmaceutical preparations and includes excipients, binders and disintegrators the choice of which depends on the specific dosage form used. Typical examples of the excipient are starch, lactose, sucrose, glucose, mannitol and cellulose; illustrative binders are polyvinylpyrrolidone, starch, sucrose, hydroxypropyl cellulose and gum arabic; illustrative disintegrators include starch, agar, gelatin powder, cellulose, and CMC. Any other common excipients, binders and disintegrators may also be employed.

In addition of the carriers described above, the pharmaceutical composition of the present invention preferably contains antioxidants for the purpose of stabilizing the effective ingredient. Appropriate antioxidants may be selected from among those which are commonly incorporated in pharmacueticals and include ascorbic acid, N-acetylcystein, L-cystein, dl-α-tocopherol, and natural tocopherol. These antioxidants are preferably used in amounts that stabilize the active compound and the weight ratio of the antioxidant to the active compound generally ranges from 0.2 to 1.5.

Formulations of the pharmaceutical composition of the present invention which are suitable for peroral administration may be provided in the form of tablets, capsules, powders, granules, or suspensions in nonaqueous solutions such as syrups, emulsions or drafts, each containing one or more of the active compounds in predetermined amounts.

The granule may be provided by first preparing an intimate mixture of one or more of the active ingredients with one or more of the auxiliary components shown above, then granulating the mixture, and classifying the granules by screening through a sieve.

The tablet may be prepared by compressing or otherwise forming one or more of the active ingredients, optionally with one or more auxiliary components.

The capsule may be prepared by first making a powder or granules as an intimate mixture of one or more of the active ingredients with one or more auxiliary components, then charging the mixture into an appropriate capsule on a packing machine, etc.

The pharmaceutical composition of the present invention may be formulated as a suppository (for rectal administration) with the aid of a common carrier such a cocoa butter.

The pharmaceutical composition of the present invention may also be formulated in a dosage form suitable for non-parenteral administration by packaging one or more active ingredients as dry solids in a sterile nitrogenpurged container. The resulting dry formulation may be administered to patients non-parenterally after being dispersed or dissolved in a given amount of aseptic water.

The dosage forms are preferably prepared from a mixture of the active ingredients, routine auxiliary components and one or more of the antioxidants listed above. If desired, the formulations may further contain one or more auxiliary components selected from among excipients, buffers, flavoring agents, binders, surfactants, thickening agents, and lubricants.

The dose of the active compound of formula (I) will of course vary with the route of administration, the severity of the disease to be treated, and the patient to be treated, but the exact dose ultimately chosen should be left to the good discretion of the doctor responsible for the treatment.

A dose whichis appropriate for the treatment of autistic disorders generally ranges from 0.1 to 50 mg/kg body weight/day, and a typical effective dose is within the range of 0.5 to 10 mg/kg body weight/day.

If a desired dose is determined, the active ingredient may be administered once a day or, alternatively, it may be administered in up to four portions daily at suitable intervals.

The active ingredient may be straightforwardly administered without being mixed with any other components. However, for several reasons, typically for the purpose of providing ease in controlling the dose level, the active compound is preferably administered in a pharmaceutical dosage form.

In addition to the compound of formula (I), the dosage formulation of the pharmaceutical composition of the present invention may contain 5-hydroxytryptophan (5HTP) and/or L-dopa (L-DOPA) as an auxiliary active ingredient. It has been observed that the combined use of these active ingredients proves even more effective in treating autism than when the active ingredient of formula (I) is used alone. If two or more active ingredients are used, their proportions are not limited to any particular value but, as guide figures, 5HTP and/or L-DOPA may be used in amounts, on a weight basis, of 0.1 to 10, preferably 0.5 to 2 parts, per 1 part of the active ingredient of formula (I).

If a pharmaceutical composition containing the mixture of active compound (I) and 5HTP and/or L-DOPA is used in treatment of autism, an appropriate dose is such that the sum of the active ingredients

ranges from 0.1 to 50 mg/kg body weight/day, preferably from 0.5 to 10 mg/kg body weight/day.

Whether the patient should be treated with a preparation containing the compound of formula (I) as the sole active ingredient or with a preparation containing both the compound (I) and 5HTP and/or L-DOPA will be decided by the good judgement of the doctor depending upon the patients age and or the severity of the disease.

As already mentioned, the active compounds which are most preferable for use in the treatment of autism are optically active L-erythro-5,6,7,8-tetrahydrobiopterin and salts thereof. They may be replaced by analogues thereof, such as DL-tetrahydrobiopterin, 1',2'-diacetyltetrahydrobiopterin, sepiapterin, 6-methyl-5,6,7,8-tetrahydropterin, 6-phenyl-5,6,7,8-tetrahydropterin, and salts thereof. It should, however, be emphasized again that from the viewpoints of nontoxicity and other factors, L-erythro-5,6,7,8-tetrahydrobiopterin which exists in vivo is most preferable. It will be interesting to note that the acute toxicity of the L-erythro-5,6,7,8-tetrahydrobiopterin dihydrochloride which was administered to rats per-orally was 2 g/kg or more, indicating the substantial absence of toxicity in this compound. The non-optically active form, DL-tetrahydrobiopterin, also presents low toxicity as demonstrated in the treatment of Parkinson's disease in Japanese Patent Public Disclosure Nos. 76086/1984 and 25323/1984, and may be used for the treatment of autism. Little acute toxicity is also found in the other compounds represented by the formula (I).

The following examples are provided for the purpose of further illustrating the present invention but are in no sense to be taken as limiting.

EXAMPLE 1

(Granules)

One part of polyvinylpyrrolidone (Kollidon 30) was dissolved in sterile purified water. The solution was uniformly mixed with 10 parts of ascorbic acid and 5 parts of L-cysteine hydrochloride. Thereafter, 10 parts of tetrahydrobiopterin dihydrochloride was added and a uniform mixture was obtained.

The resulting solution was added to 59 parts of an excipient (mannitol or lactose) and 15 parts of a disintegrator [corn starch or hydroxypropyl cellulose (LH-22)] and the kneaded mixture was granulated, dried and sieved.

EXAMPLE 2

(Tablets)

A uniform solution of tetrahydrobiopterin was prepared as in Example 1 and mixed with 58 parts of lactose and 15 parts of microcrystalline cellulose. To the mixture, one part of magnesium stearate was added and the resulting mixture was pelletized to form tablets.

EXAMPLE 3

(Capsules)

Granules as prepared in Example 1 were charged into capsules, with 0.2% magnesium stearate incorporated as a lubricant.

EXAMPLE 4

(Injection)

Tetrahydrobiopterin dihydrochloride	1.5 g
Ascorbic acid	1.5 g
L-cysteine hydrochloride	0.5 g
Mannitol	6.5 g

The above-listed components were dissolved in sterile purified water to make a volume of 100 ml. The solution was sterilized by filtration, put into vials or ampules in 1- or 2-ml portions, freeze-dried and the containers sealed.

EXAMPLE 5

(Injection)

Tetrahydrobiopterin dihydrochloride (2.0 g) was dissolved in sterile purified water in an oxygen-free atmosphere to make a volume of 100 ml. The solution was sterilized by filtration, worked up as in Example 4, and the container sealed.

EXAMPLE 6

(Suppository)

Tetrahydrobiopterin dihydrochloride	150 parts
Ascorbic acid	150 parts
L-cysteine hydrochloride	50 parts

A uniform powder prepared from these components was dispersed in 9,950 parts of cocoa butter.

EXAMPLE 7

(Granules)

Tetrahydrobiopterin dihydrochloride	5 parts
Ascorbic acid	5 parts
L-cysteine hydrochloride	2 parts

A uniform solution was prepared from these components, and added to a uniform mixture of mannitol (55 parts), polyvinylpyrrolidone (1 part), hydroxypropyl cellulose (14 parts) and 5-hydroxytryptophan (5 parts). The kneaded mixture was granulated, dried and sieved.

EXAMPLE 8

(Granules)

Tetrahydrobiopterin dihydrochloride	5 parts
Ascorbic acid	5 parts
L-cysteine hydrochloride	5 parts
Mannitol	52 parts
Polyvinylpyrrolidone (Kollidon 30)	1 part
Hydroxypropyl cellulose (LH-22)	12 parts
L-DOPA	10 parts

These components were worked up as in Example 7, followed by granulation and sieving, except that 5 parts of 5-hydroxytryptophan was replaced by 10 parts of L-DOPA.

EXAMPLE 9

(Granules)

Tetrahydrobiopterin dihydrochloride	5 parts
Ascorbic acid	5 parts
L-cysteine hydrochloride	2 parts

A uniform solution was prepared from these components, and added to a uniform mixture of 5-hydroxytryptophan (5 parts), L-DOPA (10 parts), mannitol (50 parts), polyvinylpyrrolidone (Kollidon 30) (1 part) and hydroxypropyl cellulose (LH-22) (9 parts). The kneaded mixture was granulated, dried and sieved.

The definition of autism and the legitimacy of its treatment with tetrahydrobiopterin based on biochemical observations are discussed below.

DEFINITION AND DIAGNOSIS OF AUTISM

The WHO defines infantile autism as follows: (1) it is a syndrome manifested within 30 months of birth; (2) it involves abnormal responses to visual and auditory stimulations (such as impaired understanding of language, retarded development of language, and bizarre echolalia); (3) it involves impaired social and interpersonal relations; and (4) it involves frequent stereotyped or manneristic behaviors. Patients to be treated by tetrahydrobiopterin were selected by examination to check whether their symptoms satisfied the WHO's definition of autism. Clinical symptoms and changes in abnormal behaviors were evaluated by the "Rating Scale for Abnormal Behaviors in Children" and "Children's Behavior Checklist" prepared by the "Study Group on Behavioral Disorders in Children".

RESULTS OF BIOCHEMICAL STUDIES OF AUTISM

Children with infantile autism who were selected on the basis of examination in consideration of the WHO's definition were given perorally deuterated-phenylalanine (labelled on the aromatic ring and hereinafter abbreviated as Phe-d_5) and deuterated tryptophan (labelled on the indole nucleus and hereinafter abbreviated as Trp-d_5). The results of analyses of Phe-d_5, Trp-d_5 and Tyr-d_4 (deuterated tyrosine produced as a result of metabolization of Phe-d_5 and labelled on the aromatic ring) in blood are shown in FIGS. 1 to 3, from which one can clearly see impaired transport of Trp-d_5 and Tyr-d_4 into the blood. The data in FIGS. 1 and 2 show impaired uptake of Trp-d_5 and Phe-d_5, and the Tyr-d_4 disappearance curve in FIG. 3 differs entirely from the normal curve. These results suggest that infantile autism involves abnormal transport of aromatic amino acids, and this phenomenon exist not only between blood and the intestines but also between blood and the brain. Impaired uptake of aromatic amino acids would reduce the supply of amino acids into the brain, which may lead to chronic insufficiency of serotonin and catecholamines in the brain. In the formation of serotonin, the tryptophan level could be an enzymatic activity limiting factor.

On the other hand, high blood serotonin levels in autistic children have been reported and the present inventors have confirmed that this is an indisputable fact. However, when deuterated tryptophan-3-3-d_2 (hereunder abbreviated as Trp-d_2) was administered to rats either perorally or by intraperitoneal injection, a large amount of deuterated serotonin (hereunder 5HT-d_2) was detected in the brain 30 minutes later whereas no detectable amount of 5HT-d_2 was found in the other organs checked, the only exception being the intestinal tract where a very small amount of 5HT-d_2 was detected. This indicates that although serotonin synthesis in the brain is very rapid, it is not synthesized in either platelets or plasma in the blood until several hours have passed from the administration of Trp-d_2.

Clinical observations of hish serotonin levels in autistic children would be explained as follows: impaired absorption of tryptophan by the intestinal tract causes excessive, although gradual, synthesis of serotonin in that location, from which excess serotonin is taken up by platelets. It is therefore postulated that high serotonin levels in the blood do not necessarily mean high serotonin levels in the brain, and that the reported assumption of high serotonin levels in the brain of autistic patients is quite dubious.

Therefore, on the basis of the hypothesis that the levels of serotonin and catecholamines decrease in the brain of autistic patients, the present inventors administered very small amounts of 5HTP (5-hydroxytryptophan) and L-DOPA, precursors of these amines, to the patients. The results were remarkable but the administration of these precursors required very strict dose control since any overdosage aggravated the symptoms of the patients because of the irritating action of the drugs.

The present inventors therefore reached the idea that better results would be obtained if, in addition to these amine precursors which regulate the levels of serotonin and catecholamines in the brain, tetrahydrobiopterin which is a coenzyme that is involved in the biosynthesis of these amines as their regulators was administere. The therapeutic effects of this coenzme, optionally in combination with 5HTP and/or L-DOPA, were remarkable as demonstrated in the following case studies.

Case 1: 6-year-old boy

The patient would not laugh at all and this symptom emerged about 10 months after his birth. He avoided eye contact and entirely lacked verbosity, two typical symptoms of autism. An interview with the patient revealed that he was abnormal with respect to 23 out of the 24 items in Checklist for the History of Abnormal Behaviors prepared by the "Study Group on Behavioral Disorders in Children". The patient was six years and one month old when he was brought to Musashi National Nursing Home, Tokyo, Japan. Among the most noticeable of his symptoms were: hyperkinesis, inability to interact with other people, echolaria, delayed echolaria, inability to make communicative speech, inability to verbalize, animal noise, sloppiness, and attachment to certain objects. The patient was not able to get along with classmates. Pimozide, pentoxyphylline and calcium hopantenate were either ineffective or their continued administration was impossible. His symptoms were not relieved by administration of 10 mg/kg of L-DOPA; on the contrary, this drug aggravated the patients symptoms and, hence, its application was discontinued.

Starting six weeds after the administration of L-DOPA was discontinued, 5HTP was administered at a dose of 1 mg/kg/day. Eight weeks after the commencement of 5HTP administration, the frequency of echolalia decreased and the patient started to utter normal words and sentences. In the ninth week, 2 mg/kg of L-DOPA was administered in addition to 5HTP; the

patient acquired the ability to lead a school life and interact with other children but no further improvements were attained. Therefore, the administration of L-erythro-5,6,7,8-tetrahydrobiopterin (hereunder BPH_4) was incorporated in the regimen.

When 2.5 mg/kg/day of BPH_4 was administered in addition to 5HTP and L-DOPA, the patient got excited for a while but at day he became less excitable, and thereafter, he was well disciplined and could play with other children. One week later, the administration of 5HTP and L-DOPA was discontinued and only BPH_4 was given at a dose of 2.5 mg/kg/day, but the symptoms of the patients were further relieved.

One week after the administration of BPH_4 alone, 5HTP (1 mg/kg/day) was again administered in combination with 1 mg/kg/day of BPH_4. After one week of the combined administration of BPH_4 and 5HTP, the patient became normal in terms of understanding instructions, playing, sleeping and interacting with his family. He even became able to attend school, walk alone, and go out of his home in the same way as normal children. In view of such pronounced improvements, the administration of BPH_4 was discontinued at week 6 and only 5HTP was administered thereafter. Then, the patient feel again into hyperkinesis, requently uttered animal noises, and refused to stop this undesirable behavior when ordered to do so verbally. His symptoms were further aggravated when the administration fo 5HTP was suspended.

Following a suspension of about 8 weeks in the administration of drugs, the administration fo BPH_4 above (1 mg/kg/day) was resumed. The patient still suffered from hyperkinesis and would laugh to himself. However, his ability to understand language improved and he became able to interact and converse with othere people, making appropriate responses to them (FIG. 4 and Table 1).

years and 8 months old, the patient was unable to speak, did not obey verbal instructions, was hyperactive lacked outward expressiveness, and showed abnormal attachment to objects. Pimozide, pentoxyphylline and calcium hopantenate were ineffective. The patient was five years old when he was hospitalized at Municipal Children's Health Center, Osaka, Japan.

The patient was given L-DOPA at a dose of 10 mg/kg/day. At day 10, he showed increasing interest in other people and manifested other responses which were, however, by no means pronounced.

At day 11 and afterward, both 5HTP (1 mg/kg/day) and L-DOPA (10 mg/kg/day) were administered. About two weeks after this combined drug regimen, the patient started to respond to other person's calls and to verbalize his feelings. However, the improvement was not appreciable and the drug treatment was suspended after four weeks.

In the fifth week and afterward, the patient was given BPH_4 (1 mg/kg/day) alone. The patient's interpersonal transactional mode improved and he responded to selected persons, enabling others to infer his emotions. He expressed his emotions with words and he showed increasing interest in exercising the whole body and playing ball. The overall improvement was pronounced.

In the seventh week and afterward, the patient was given both BPH_4 (1 mg/kg/day) and 5HTP (1 mg/kg/day). He showed better understanding of language and became able to develop a social life with other children. He also learned to count numbers and be patient. The improvements were great.

When the drug application was discontinued in the 12th week, the patient gradually post his patience, got easily excited and showed a tendency to avoid eye contact. During a 10-day intermission, the symptoms of the patient became seriously aggravated.

After the 10-day intermission, the administration of

TABLE 1

	Changes in Symptoms of Case 1 at Various Stages of Drug Administration								
	Before Treatment	L-DOPA (10)	5HTP (2)	5HTP (1) + L-DOPA (2)	BH_4 (2.5) + 5HTP (1) + L-DOPA (2)	BH_4 (2.5)	BH_4 (1) + 5HTP (1)	5HTP (2)	BH_4 (1)
Hyperkinesis	+++	+++	++	++	+++	++	±	++	+
Emotional Lability	+++	+++	++	++	++	++	+	+	+
Attachment	+++	+++	+~++	+~++	+~++	+~++	+	++	+
Refusal	++	++	+	+	+	+	+	++	+
Poor verbalization	++	++	+	+	+~++	+	±	++	+
Echolaria	+++	+++	+	+	+	+	±	++	±
Poor understanding	++	++	+	+	+	+	±	++	+
Lack of communication	+++	+++	++	+	+	+	+	+~++	+
Inability to play	+++	+++	++	+	+	±	±	+	±
Poor adaptability	+++	+++	++	+	+	±	±	+	+
Stereotyped behavior	++	++	+	+	++	+~++	+	+~++	+
Insistence on the preservation of sameness	+++	+++	+	+	+	+	+	+~++	+
Trouble in sleeping	+	+++	±	+~++	+~++	±	−	+	−
Rating of improvement		aggravated	slightly improved	improved	slightly aggravated	improved	markedly improved	aggravated	improved

+++: abnormalities very pronounced;
++: abnormalities pronounced;
+: abnormalities slight;
−: abnormalities unnoticeable

Case 2: 5-year-old boy

Since earliest infancy, the patient manifested such symptoms as the avoidance of eye and human contact. Interview with the patient revealed that he was abnormal with respect to 22 out of the 24 items in the Children Behavior Checklist prepared by the "Study Group on Behavioral Disorders in Children". When he was 4

BPH_4 (1 mg/kg/day) and 5HTP (1 mg/kg/day) was resumed. The improvement in the patient's ability to understand language, interact with other people, and respond to other person's calls was so remarkable that the patient almost looked like a normal child (FIG. 5 and Table 2).

TABLE 2

	Changes in Symptoms of Case 2 at Various Stages of Drug Administration						
	Before Treatment	L-DOPA (10)	5HTP (1) + L-DOPA (10)	BH_4 (1)	BH_4 (1) + 5HTP (1)	After Intermission	BH_4 (1) + 5HTP (1)
Hyperkinesis	++	++	+	+	±	+	−
Emotional Lability	+++	+	±	±	±	++	±
Attachment	+++	++	++−+	+	+	+	+
Refusal	+++	++	+	−	−	+	−
Poor verbalization	+++	+++	+++	+	+	++	+
Echolaria				+	+	+	±
Poor understanding	+++	++	+	±	±	+	±
Lack of communication	+++	+	+	±	±	++	±
Inability to play	+++	++	+	+	±	+	±
Poor adaptability	+++	+	+	+	±	+	±
Stereotyped behavior	+++	+++	++	+	+	+	±
Insistence on the preservation of sameness	+++	+++	++	+	+	+	±
Trouble in sleeping	+	+	−	−	−	−	−
Rating of improvement		slightly improved	improved	markedly improved	drastically improved	aggravated	drastically improved

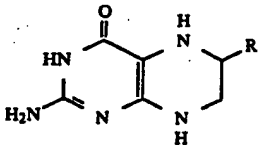
+++: abnormalities very pronounced;
++: abnormalities pronounced;
+: abnormalities slight;
−: abnormalities unnoticeable

The above-described cases are totally representative of autistic patients and were objectively supported by the Checklist of Medical History and the "Rating Scale for Present Abnormal Behaviors in Children" prepared by the "Study Group on Behavioral Disorders in Children". The present inventors administered BPH_4 to these representative cases and attained strikingly good results. The ability of BPH_4 to alleviate the symptoms of autism was also demonstrated by the fact that the patients got worse as a result of discontinuation of drug treatment and that they changed for better when drug administration was resumed.

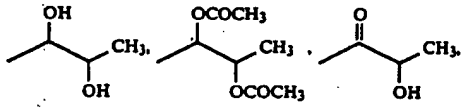
Remarkable effects were attained even when BPH_4 was administered alone, but the two case studies demonstrate that satisfactory results could also be obtained by using BPH_4 in combination with 5HTP and/or with L-DOPA capable of increasing the levels of catecholamines in the brain.

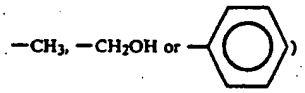
We claim:

1. A method for ameliorating disorder of infantile autism, wherein an autistic child receives a pharmaceutical composition comprising a compound of the formula:



(wherein R is





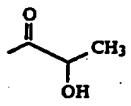
—CH_3, —CH_2OH or)

or a salt thereof as an effective ingredient, at a dose wherein the amount of the effective ingredient received is in the range of 0.1 to 50 mg/kg body weight/day together with a pharmaceutically acceptable carrier.

2. A method according to claim 1, wherein the pharmaceutical composition further contains an anti-oxydizing agent selected from the group comprising ascorbic acid, N-acetylcysteine, L-cysteine, dl-α-tocopherol and natural tocopherol.

3. A method according to claim 1, wherein the amount of the effective ingredient received is in the range of 0.5 to 10 mg/kg body weight/day.

4. A method according to claim 1, wherein R is



5. A method according to claim 1, wherein the effective ingredient is L-erythro-5,6,7,8-tetrahydrobiopterin.

* * * * *

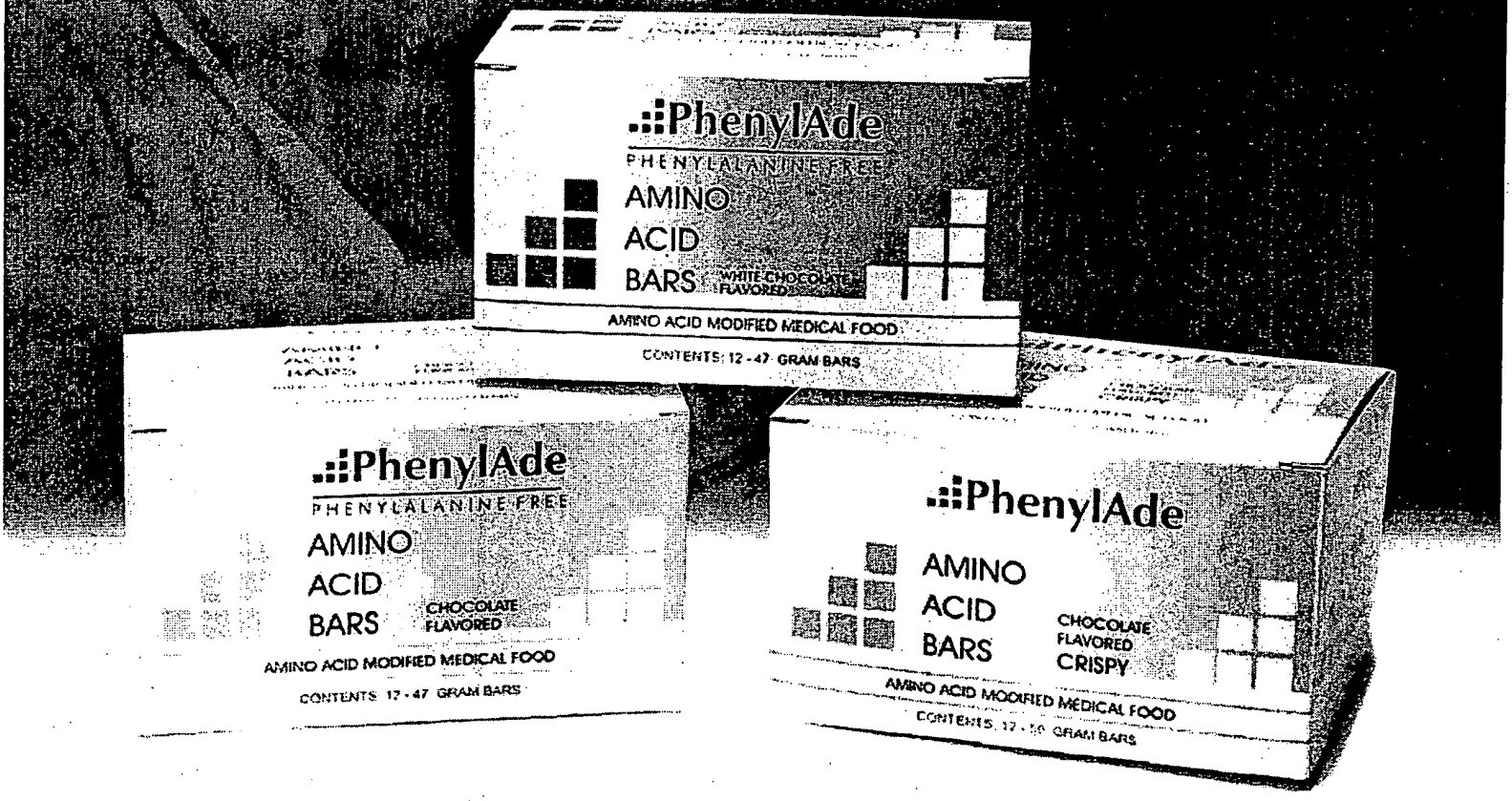
EXHIBIT P

PhenylAde®

MEDICAL FOODS FOR THE DIETARY MANAGEMENT OF PHENYLKETONURIA

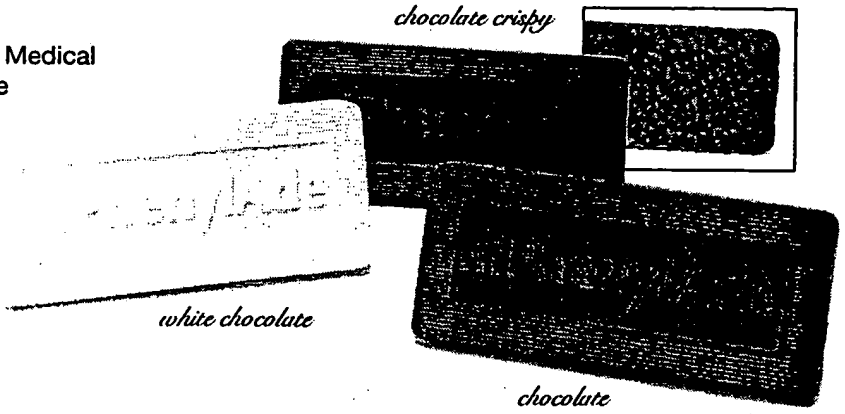
PRODUCT GUIDE

Amino Acid Bars



Amino Acid Bars

PhenylAde Amino Acid Bars are an innovative Medical Food intended to provide an alternative source of protein, fat and carbohydrate for the dietary management of PKU. PhenylAde Amino Acid Bars can supplement all or part of the protein prescription when traditional liquid formula is insufficient or not tolerated. Available in 3 delicious flavors, PhenylAde Amino Acid Bars look, taste and smell like real chocolate.



Combine with PhenylAde Drink Mix and Amino Acid Blend* for a flexible PKU Diet.*

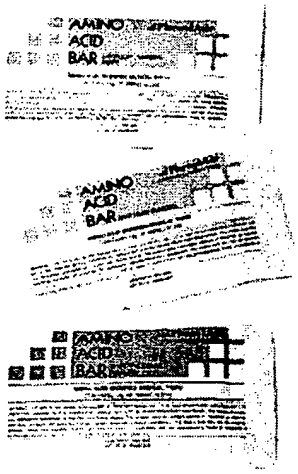
APPLIED NUTRITION Corporation

PhenylAde®

Amino Acid Bars

Features
- Supplies a full 10 grams of Protein Equivalent
- Individually wrapped bars
- Looks, smells and tastes like a real chocolate bar

Directions for Use
For use in the dietary management of Phenylketonuria under the medical supervision of a physician. PhenylAde Amino Acid Bars can be used by children, teenagers, adults and pregnant women and must be taken in combination with low protein foods. A vitamin/mineral supplement may be necessary to complete a nutritionally balanced diet. PhenylAde Amino Acid Bars do not contain added vitamins or minerals so that the attending physician can prescribe the appropriate vitamin/mineral supplement as part of a total diet plan.
NOT FOR INFANTS UNDER ONE YEAR OLD.



Storage
Store in a cool dry place, can be refrigerated if desired. Avoid direct heat or sunlight.

Warning
Do not heat medical food or try to use in cooking.

Order Information

Pharmacists:
All PhenylAde products can be special ordered from your wholesaler as a "drop-ship item" or they can be ordered directly from the manufacturer, Applied Nutrition Corp. For customer service CALL TOLL FREE **(800) 605-0410.**

Metabolic Professionals:
Contact **info@medicalfood.com** or call **(800) 605-0410** for samples and additional product information.

Retail Customers:
All PhenylAde products are available through your retail pharmacy. Please allow sufficient time for your first prescription to be shipped and fulfilled. To order directly from the manufacturer please call **(800) 605-0410** between 8:30 am and 5:00 pm EST or FAX your order anytime to **(973) 361-6707.**

PhenylAde Amino Acid Bars *Packed: 12 Bars Per Case*	Product Code	Reimbursement Code
White Chocolate Flavored	9570	00847-0957-06
Chocolate Crispy Flavored	9580	00847-0958-06
Chocolate Flavored	9590	00847-0959-06

Nutrition

AMINO ACID BARS

	Chocolate & White Chocolate	Chocolate Crispy*
Calories	270	270
Serving Size	1 bar (47g)	1 bar (50g)
Protein Equivalent, g	10	10
Source	L-Amino Acids	L-Amino Acids
Amino Acids, mg		
L-Alanine	938 mg	987 mg
L-Arginine	799	851
L-Asparagine	1397	1477
L-Carnitine	10	10
L-Cystine	134	144
L-Glutamine	1547	1647
Glycine	604	642
L-Histidine	249	266
L-Isoleucine	399	427
L-Leucine	888	944
L-Lysine	908	957
L-Methionine	265	282
L-Phenylalanine	0	8*
L-Proline	604	639
L-Serine	996	1048
Taurine	30	31
L-Threonine	470	500
L-Tryptophan	150	159
L-Tyrosine	924	976
L-Valine	669	711
Fat, g	18	17
Source	Cocoa Butter	Cocoa Butter
Saturated Fat	11	10
Monounsaturated Fat	6	6
Polyunsaturated Fat	1	1
Linoleic Acid, g	.25	.25
Linolenic Acid, g	.07	.07
Cholesterol, mg	0	0
Carbohydrate, g	17	19
Source	Sucrose	Sucrose, Crisp Rice

*Crisp rice adds a small amount of protein including Phenylalanine.

Made in USA by:

APPLIED NUTRITION *Corporation*
METABOLIC NUTRITION SUPPORT

273 Franklin Road
Randolph, NJ 07869
(800) 605-0410
www.medicalfood.com

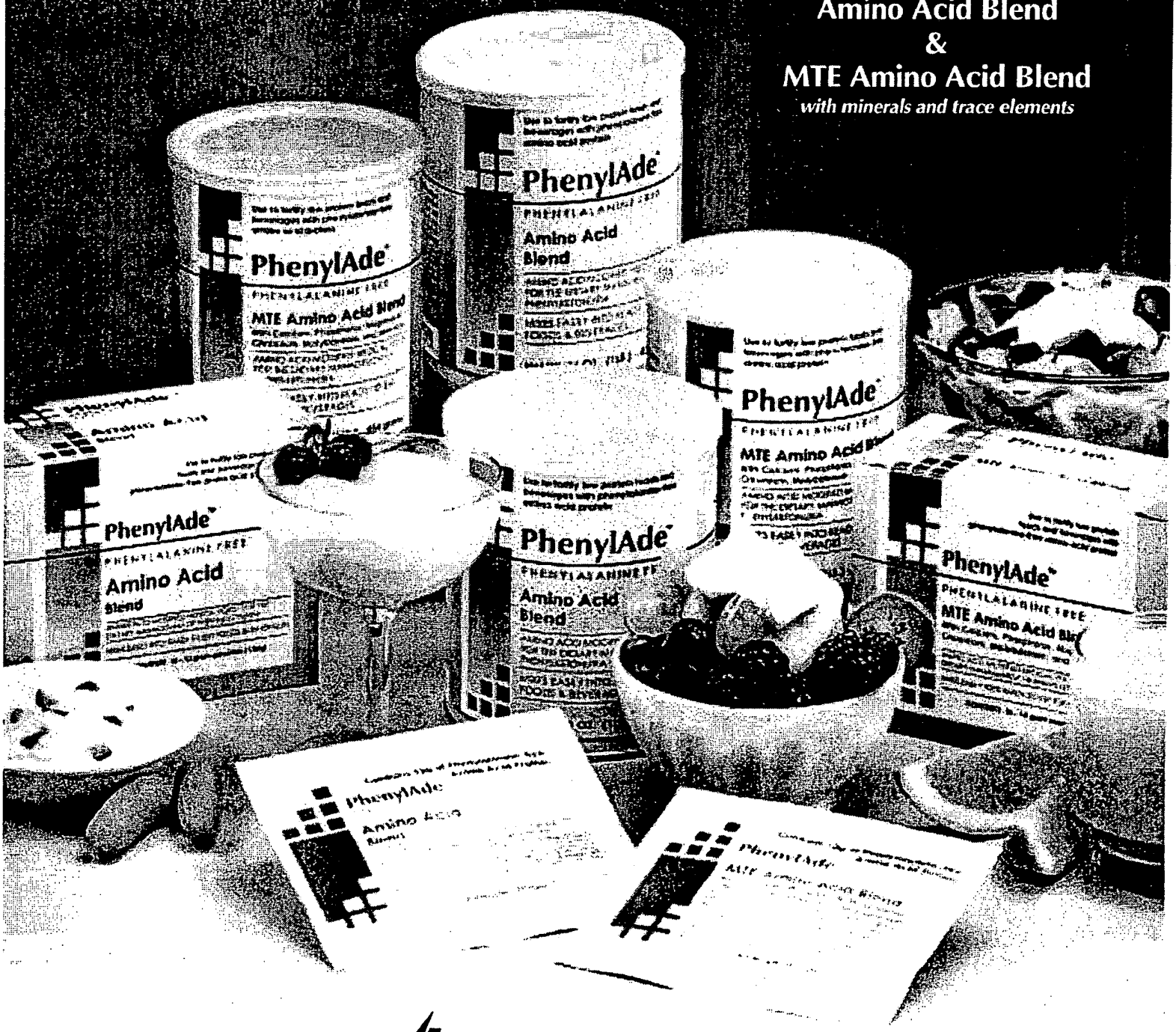
EXHIBIT Q

PhenylAde™

MEDICAL FOODS FOR THE DIETARY MANAGEMENT OF PHENYLKETONURIA

PRODUCT GUIDE

Amino Acid Blend
&
MTE Amino Acid Blend
with minerals and trace elements



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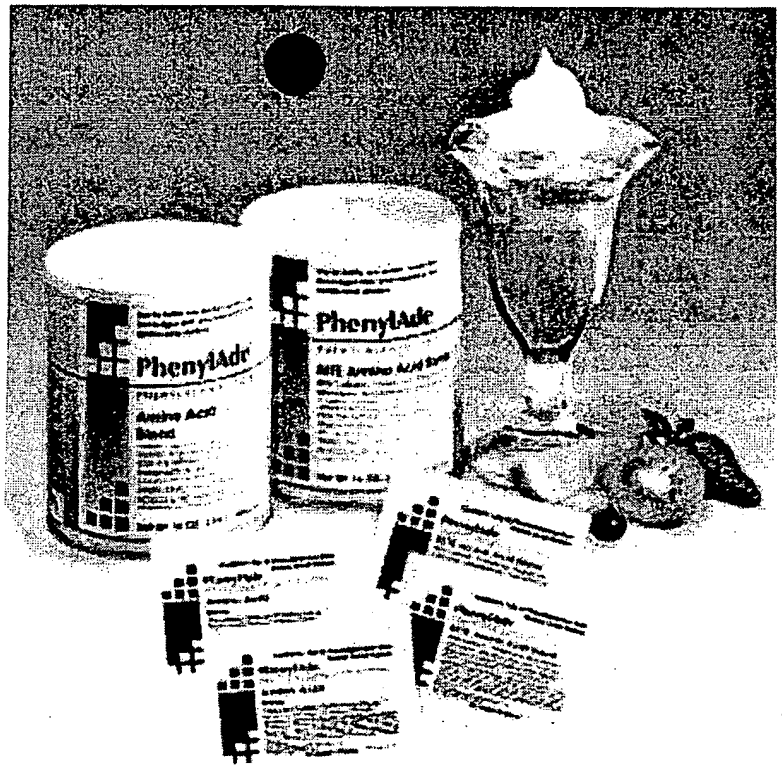
PhenylAde™

AMINO ACID BLENDS



PhenylAde Amino Acid Blends are specialized mixtures of essential and non-essential amino acids designed for the dietary management of Phenylketonuria. As modular medical foods, PhenylAde Amino Acid Blends can provide flexibility in diet prescription, thus improving diet adherence and reducing formula fatigue.

The development of this product was inspired by the need to add variety to the approach of diet management in a realistic and appetizing manner. Research published in the Journal of Inherited Metabolic Disease[1] proved PhenylAde Amino Acid Blends are an effective alternative to the typical "all-in-one" medical foods. Study participants were prescribed different combinations of nutritionally complete and incomplete medical foods. PhenylAde Amino Acid Blend was rated the highest on a food sensory scale (see diagram below), and at the end of the five-year study more people remained on a diet plan which included PhenylAde Amino Acid Blend in comparison to the alternative medical foods. This approach to a PKU-diet has been recognized as safe due to the results of this study. Throughout the five-year period, normal growth was maintained.

FEATURES:

- **Mixes easily into ready-to-eat foods and beverages.**
- **PhenylAde Amino Acid Blends can be used to fortify PKU medical formulas, in some cases decreasing the volume necessary to consume.**
- **Convenient pre-measured pouches.**
- **Each scoop or pouch of PhenylAde Amino Acid Blend provides 10 grams of protein equivalent making short and long-term diet adjustments easier.**
- **A serving of PhenylAde Amino Acid Blend can be substituted for a serving of other PhenylAde products for variety and flexibility.**

INITIAL MEDICAL FOOD SENSORY EVALUATION RATINGS

Rating Scale:
5 = like extremely
4 = like
3 = neither like or dislike
2 = dislike
1 = dislike extremely

	OVERALL	TASTE	ODOR	TEXTURE	APPEARANCE
PhenylAde Amino Acid Blend Added To Lemon Pudding	4.9	4.9	4.8	4.8	4.7
PhenylAde Amino Acid Blend Added To Tomato Sauce	4.7	4.5	4.5	4.5	4.6
PhenylAde Amino Acid Bar	4.0	4.0	4.5	4.0	4.8
PhenylAde Drink Mix Vanilla Flavored	3.5	3.3	3.9	3.2	3.1

[1]Prince, A.P., McMurry, M.P. and Buist, N.R.M., Journal of Inherited Metabolic Disease, August 1997

NUTRITION:

	AMINO ACID BLEND	MTE AMINO ACID BLEND
Calories	42	42
Serving Size, g	13 (per scoop or pouch)	13 (per scoop or pouch)
Protein Equivalent, g	10	10
Source	L-Amino Acids	L-Amino Acids
Amino Acids, mg		
L-Alanine	1018 mg	992 mg
L-Arginine	866	845
L-Asparagine	1516	1477
L-Carnitine	11	10
L-Cystine	146	142
L-Glutamine	1679	1635
Glycine	655	638
L-Histidine	271	264
L-Isoleucine	433	422
L-Leucine	964	939
L-Lysine	986	960
L-Methionine	287	280
L-Phenylalanine	0	0
L-Proline	655	638
L-Serine	1081	1053
Taurine	33	31
L-Threonine	509	497
L-Tryptophan	163	159
L-Tyrosine	1002	976
L-Valine	726	707
Minerals		
Calcium, mg	0	59 mg
Phosphorus, mg	0	59 mg
Magnesium, mg	0	16 mg
Molybdenum, µg	0	3µg
Chromium, µg	0	3µg
Selenium, µg	0	1µg

Use this chart as a guide to develop personalized diet plans. The nutrient composition of all PhenylAde brand products is unique, but at the same time all provide 10 grams of protein equivalent. This allows for easy prescription adjustments and implementation into a variety of lifestyles and stages of life.

PhenylAde Amino Acid Blends are an appropriate medical food for many individuals. Young children, teenagers, adults, and pregnant women can benefit from the flexibility and fortification characteristics of PhenylAde Amino Acid Blends.

DAILY DIET STRATEGIES

	PhenylAde Amino Acid Blend	PhenylAde Amino Acid Blend *PLUS* PhenylAde Drink Mix*	PhenylAde Amino Acid Blend *PLUS* PhenylAde Amino Acid Bars*
6 YEAR OLD *protein requirement: 30 g/day*			
PhenylAde Amino Acid Blend	3 scoops or pouches	1 scoop or pouch	2 scoops or pouches
PhenylAde Drink Mix	0	2 scoops	0
PhenylAde Amino Acid Bars	0	0	1 bar
Totals from medical foods:	*126 kcal, 30 g protein equivalent*	*370 kcal, 30 g protein equivalent*	*344 kcal, 30 g protein equivalent*
13 YEAR OLD *protein requirement: 40 g/day*			
PhenylAde Amino Acid Blend	4 scoops or pouches	2 scoops or pouches	2 scoops or pouches
PhenylAde Drink Mix	0	2 scoops	0
PhenylAde Amino Acid Bars	0	0	2 bars
Totals from medical foods:	*168 kcal, 40 g protein equivalent*	*412 kcal, 40 g protein equivalent*	*604 kcal, 40 g protein equivalent*
18 YEAR OLD *protein requirement: 50 g/day*			
PhenylAde Amino Acid Blend	5 scoops or pouches	3 scoops or pouches	3 scoops or pouches
PhenylAde Drink Mix	0	2 scoops	0
PhenylAde Amino Acid Bars	0	0	2 bars
Totals from medical foods:	*210 kcal, 50 g protein equivalent*	*454 kcal, 50 g protein equivalent*	*646 kcal, 50 g protein equivalent*
MATERNAL PKU *protein requirement: 60 g/day*			
PhenylAde Amino Acid Blend	6 scoops or pouches	3 scoops or pouches	3 scoops or pouches
PhenylAde Drink Mix	0	3 scoops	0
PhenylAde Amino Acid Bars	0	0	3 bars
Totals from medical foods:	*252 kcal, 60 g protein equivalent*	*618 kcal, 60 g protein equivalent*	*906 kcal, 60 g protein equivalent*

** Please see the appropriate PhenylAde Product Guide for complete nutrition information on these products.*

FOR EXAMPLE: If your are currently using a product which contains 25 grams of protein equivalent per 100 grams of powder (recommended dilution of 50 grams powder to 1 cup water), and your protein prescription is for 60 grams/day, it can be assumed that your formula for one day will be 240 grams of powder mixed in 5 cups of water.

To modify this you could:

1. **Get half of your protein requirement from PhenylAde Amino Acid Blend.** 120 grams of your traditional formula + 3 scoops or 3 pouches (39 grams) of PhenylAde Amino Acid Blend and mix with 2 1/2 cups of water.

   OR Choose 3 food items throughout the day to mix your PhenylAde Amino Acid Blend into (i.e. salad dressing, lo-pro pudding, ketchup).

2. **Get all of your protein from PhenylAde Amino Acid Blend.** Mix 6 scoops or 6 pouches of PhenylAde Amino Acid Blend into 4 cups of your favorite beverage (i.e. Kool-Aid, Tang, Lemonade).

   OR Mix 3 scoops or pouches of PhenylAde Amino Acid Blend in 2 or more cups of your beverage of choice and pick 3 food items throughout the day to mix your blend into.

These are only a few examples of how PhenylAde Amino Acid Blend can be incorporated into a healthy diet. It is easy to adjust the diet when one serving of product equals 10 grams of protein equivalent. You can use any combination of the above diet strategies. PhenylAde Amino Acid Blends allow you to be more flexible, however, drinking formula at regular intervals, consuming the entire portion of medical food, and following a low protein diet remains important.

PhenylAde Amino Acid Blend is available in two forms: PhenylAde Amino Acid Blend and PhenylAde MTE Amino Acid Blend. PhenylAde MTE Amino Acid Blend contains minerals and trace elements that are typically lacking in a low protein diet: calcium, phosphorus, magnesium, molybdenum, chromium, and selenium. These nutrients are not available in significant quantities in most over the counter vitamin/mineral supplements. A vitamin/mineral supplement may be necessary to complete a nutritionally balanced diet. PhenylAde Amino Acid Blends do not contain vitamins and minerals so that the attending physician can prescribe the appropriate vitamin/mineral supplement as part of a total diet plan.

INDICATIONS:

- **Not for infants under one year old. Not for parenteral use.**
- **For use only in the dietary management of Phenylketonuria under the medical supervision of a physician.**
- **Amino Acid Blends should be used in conjunction with a low protein diet and an appropriate vitamin/mineral supplement.**

MIXING INFORMATION AND DIRECTIONS FOR USE:

Measure prescribed amount of PhenylAde Amino Acid Blend and add to a single portion of desired low protein food or beverage. It is important that the appropriate serving size is established and the entire portion of food is consumed to ensure that all of the Amino Acid Blend has been ingested.

Do not heat medical food or try to use in cooking.

For optimal acceptability consider foods that a reasonable portion can be mixed with a serving of PhenylAde Amino Acid Blend. For example, one scoop or pouch mixes nicely with 1/4 cup of ketchup, dip/salad dressing, applesauce, baby food, or low protein pudding. Do not mix into only water. Amino Acid Blend is intended to be added to ready-to-eat foods and beverages.

DRY MEASURE EQUIVALENTS:

1 Tbs. =	9.5g blend =	8g Protein Equivalent =	32 Calories
1 SCOOP* OR POUCH =	13g blend =	10g Protein Equivalent =	42 Calories
1/4 cup =	40g blend =	33.5g Protein Equivalent =	135 Calories
1/2 cup =	80g blend =	67g Protein Equivalent =	269 Calories

* SCOOP ENCLOSED IN EACH CAN

ORDER INFORMATION:

Product	Product Code	Reimbursement Code
PhenylAde Amino Acid Blend	9500	00847-0950-00
PhenylAde Amino Acid Blend Pouches	95004	00847-0950-04
PhenylAde MTE Amino Acid Blend	9596	00847-0959-60
PhenylAde MTE Amino Acid Blend Pouches	95964	00847-0959-64

Pharmacists: All PhenylAde products can be special ordered from your wholesaler as a "drop-ship item" or they can be ordered directly from the manufacturer, Applied Nutrition Corp.
For customer service CALL TOLL FREE 800-605-0410.

Metabolic Professionals: Contact **info@medicalfood.com** or call 800-605-0410 for samples and additional product information.

Retail Customers: All PhenylAde products are available through your retail pharmacy. Please allow sufficient time for your first prescription to be shipped and fulfilled. To order directly from the manufacturer please call 800-605-0410 between 8:30 am and 5:00 pm EST or FAX your order anytime to 973-361-6707.

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RECIPES:

WALDORF SALAD

3 cups (360 gm) diced apples
1/2 cup (53 gm) diced celery
1/4 cup (40 gm) raisins
1/2 cup (20 gm) mini-marshmallows
2 tablespoons Miracle Whip Salad Dressing
2 tablespoons Cool Whip
4 scoops or pouches Amino Acid Blend

Mix apples, celery, raisins and marshmallows in a large bowl. Blend together the Miracle Whip, Cool Whip and Amino Acid Blend: mix into salad. Chill.
Completed salad can be refrigerated for up to 72 hours.
Yield: 4 1/4 cups (514 gm).

	Phenylalanine (mg)	Protein (gm)	Calories	PhenylAde Amino Acid Protein Equivalent (gm)
Per Recipe	76	2.9	689	40
Per 1/2 cup (60 gm) serving	9	0.3	108	4.7

CREAMY DIP

1/2 to 1 teaspoon Hidden Valley Salad Dressing Mix
3 tablespoons Cool Whip or Rich Whip Topping
1/2 to 1 teaspoon Hidden Valley Salad Dressing Mix from a 1 oz. package
1 teaspoon Lemon Juice
1 scoop or pouch Amino Acid Blend

Mix all ingredients in a small bowl. Chill.
Completed dip can be refrigerated for up to 72 hours.
Yield: 1/2 cup (60 gm).

	Phenylalanine (mg)	Protein (gm)	Calories	PhenylAde Amino Acid Protein Equivalent (gm)
Using Rich Whip				
Per Recipe	35	0.7	390	10
Per Tablespoon	4	0.1	49.25	1.25
Using Cool Whip				
Per Recipe	44	0.8	357	10
Per Tablespoon	5	0.1	44.25	1.25

SARAH'S LEMONADE

1 packet un-sweetened Kool-Aid
5 packets Sweet 'N Low
32 ounces of cold water and ice to taste
3 scoops or pouches Amino Acid Blend

Mix Kool-Aid, Sweet 'N Low and Amino Acid Blend. Add water and ice.
Mix until powders are dissolved.

	Phenylalanine (mg)	Protein (gm)	Calories	PhenylAde Amino Acid Protein Equivalent (gm)
Per Recipe	0	0	0	30
Per 1/2 cup (60 gm) serving	0	0	0	7.5

EASY STIR-IN IDEAS

Add 1 scoop or pouch Amino Acid Blend to 1/2 cup salsa

Add 1 scoop or pouch Amino Acid Blend to 1/2 cup bottled French Dressing

Add 1 scoop or pouch Amino Acid Blend to 1/2 cup cherry pie filling and serve with whipped topping

Try adding to your favorite low protein pudding, baby food or applesauce

Remember to count the phenylalanine content of the ingredients you use.

Waldorf Salad and Creamy Dressing recipes are courtesy of; Schuett, Virginia E. LOW PROTEIN COOKERY FOR PHENYLKETONURIA, 3rd Edition.

Nutrition Tip:

ADD 1 SCOOP OR POUCH OF PHENYLADE AMINO ACID BLEND TO TRADITIONAL FORMULA TO INCREASE THE AMINO ACID PROTEIN EQUIVALENT BY 10 GRAMS!

TRY OTHER PHENYLADE PRODUCTS!

EXHIBIT R

US 20020052374A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2002/0052374 A1

Rabelink et al. (43) Pub. Date: May 2, 2002

(54) PHARMACEUTICAL PREPARATION CONTAINING AT LEAST FOLIC ACID OR A FOLATE AND TETRAHYDROBIOPTERIN (BH4) OR DERIVATIVES THEREOF USED FOR TREATING OR PREVENTING CARDIOVASCULAR OR NEUROLOGICAL DISORDERS BY MODULATING OF THE ACTIVITY OF NITRIC OXIDE SYNTHASE (NOS)

(76) Inventors: Ton J. Rabelink, Utrecht (NL); Rudolf Moser, Schaffhausen (CH)

Correspondence Address:
MILLEN, WHITE, ZELANO & BRANIGAN, P.C.
2200 CLARENDON BLVD.
SUITE 1400
ARLINGTON, VA 22201 (US)

(*) Notice: This is a publication of a continued prosecution application (CPA) filed under 37 CFR 1.53(d).

(21) Appl. No.: 09/588,301

(22) Filed: Jun. 7, 2000

Publication Classification

(51) Int. Cl.[7] A61K 31/495; A61K 31/50; A01N 43/58; A01N 43/60

(52) U.S. Cl. 514/250

(57) ABSTRACT

The invention relates to the use of at least folic acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof for treating or preventing cardiovascular or neurological disorders by modulation of the activity of nitric oxide synthase (NOS).

The present invention also relates to the use of at least folio acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof for the production of a pharmaceutical preparation suitable for influencing the nitric oxide (NO) level, particularly by modulation of the activity of nitric oxide synthase (NOS) by reducing superoxide (O_2) production and enhancing nitric oxide (NO) synthesis. This effect occurs in absence of negative changes in other risk factors, e.g. lipids, blood pressure and homocysteine. Clinical areas of application include all anomalies of the nitric oxide level, particularly the prevention and treatment of cardiovascular and of neurological disorders.

The present invention also relates to pharmaceutical preparations comprising at least one compound selected from the group consisting of 5-formyl-(6 S)-tetrahydrofolic acid, 5-methyl-(6 S)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5-formimino-(6 S)-tetrahydrofolic acid or (6 S)-tetrahydrofolic acid or pharmaceutically compatible salts thereof, together with tetrahydrobiopterin (BH_4) and with pharmaceutically compatible active and adjuvant substances, such as L-arginine, for influencing the nitric oxide (NO) level,

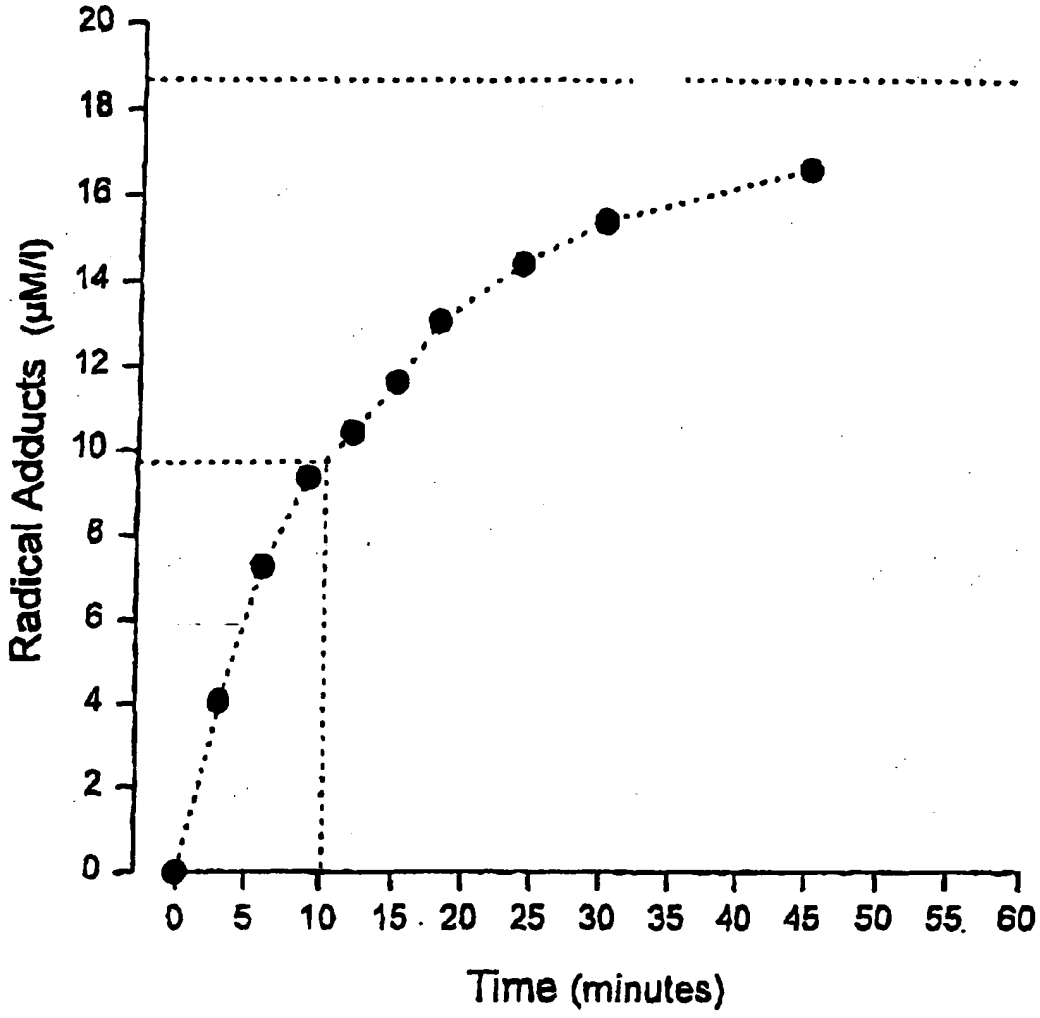

Figure 1

Time-curve of EPR intensity for HX/XO

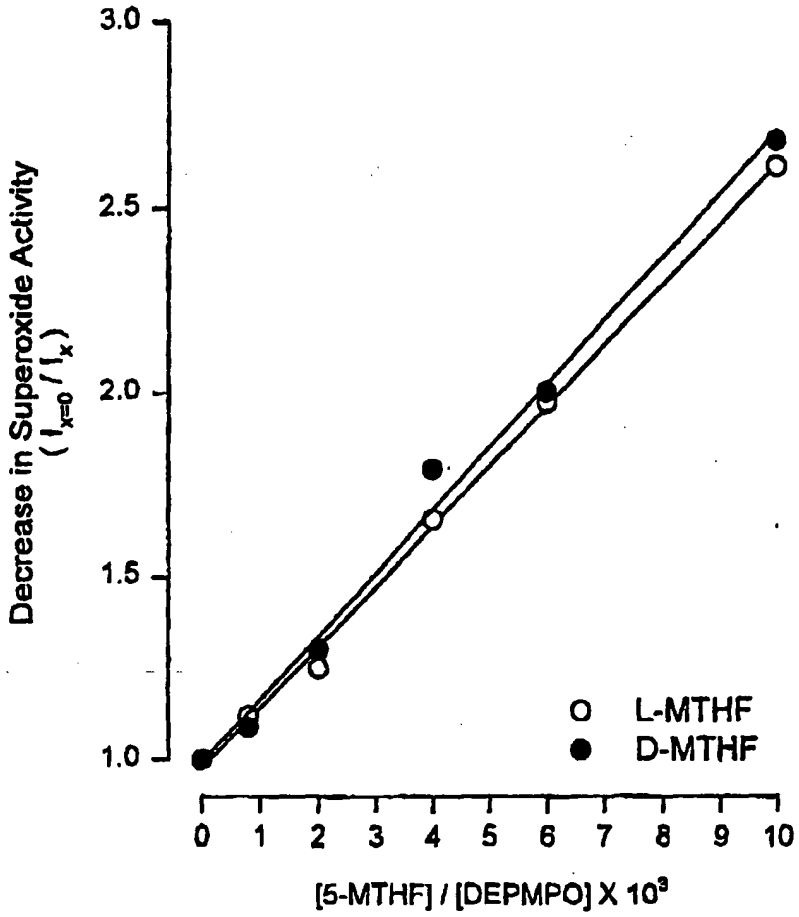

Figure 2

Competitive superoxide trapping by 5-methyl-(6S)- and –(6R)-tetrahydrofolic acid in HX/XO

From the slope of the curves it follows that the superoxide trapping rates for 5-methyl-(6S)-tetrahydrofolic acid (open circles) and 5-methyl–(6R)-tetrahydrofolic acid (solid circles) are similar and about 175 times the superoxide trapping rate of DEPMPO

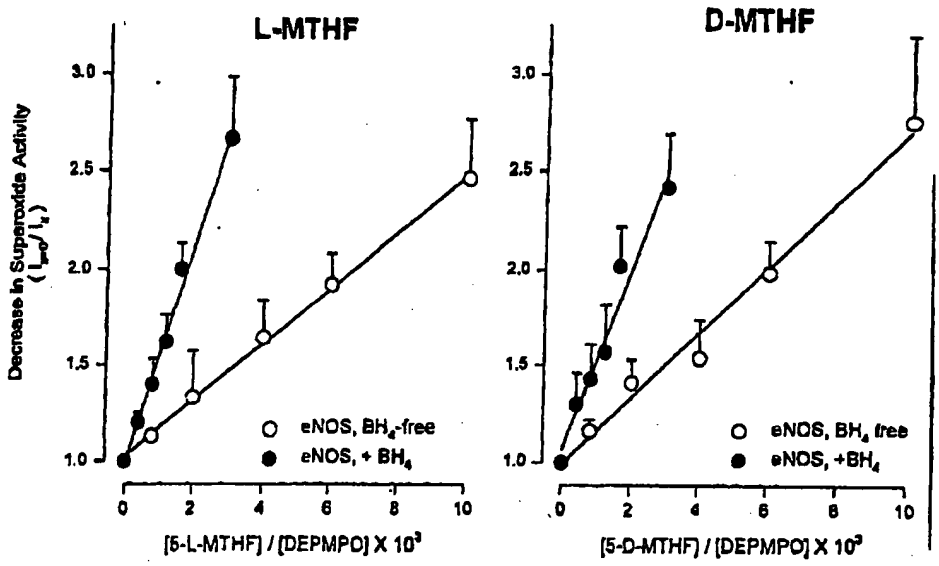

Figure 3

Competitive superoxide trapping by 5-methyl-(6S)- and –(6R)-tetrahydrofolic acid in eNOS

The slope of the curve is much steeper for pterin-repleted (solid circles) than for pterin-free (open circles) eNOS, both for 5-methyl-(6S)- as well as –(6R)-tetrahydrofolic acid ($p<0.05$ pterin-repleted vs. pterin-free eNOS). This shows that 5-methyltetrahydrofolic acid interferes with enzymatic superoxide production by pterin-repleted eNOS.

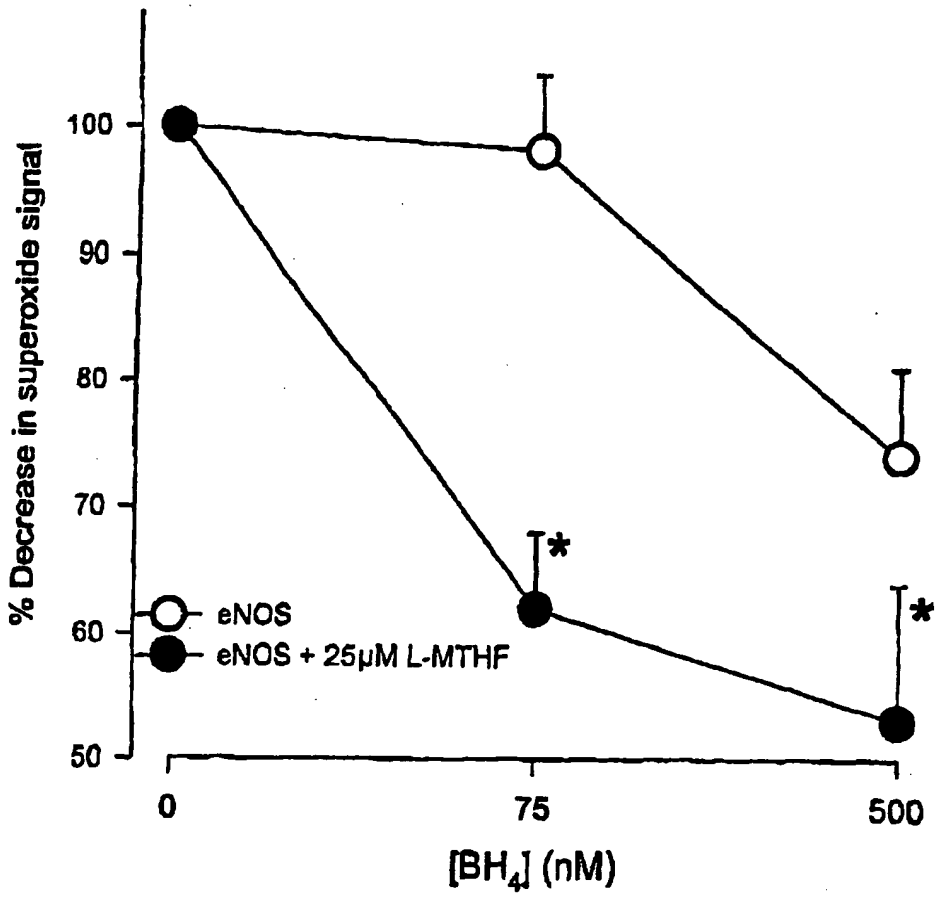

Figure 4

Effect of tetrahydrobiopterin (BH_4) on superoxide production by eNOS in presence and absence of 5-methyl-(6S)-tetrahydrofolic acid

BH_4 induces a dose-dependent decrease in radical-adduct formation (open circles). Preincubation with 5-methyl-(6S)-tetrahydrofolic acid (25 μM) significantly enhances the BH_4-associated decrease in radical adduct formation by eNOS (closed circles).

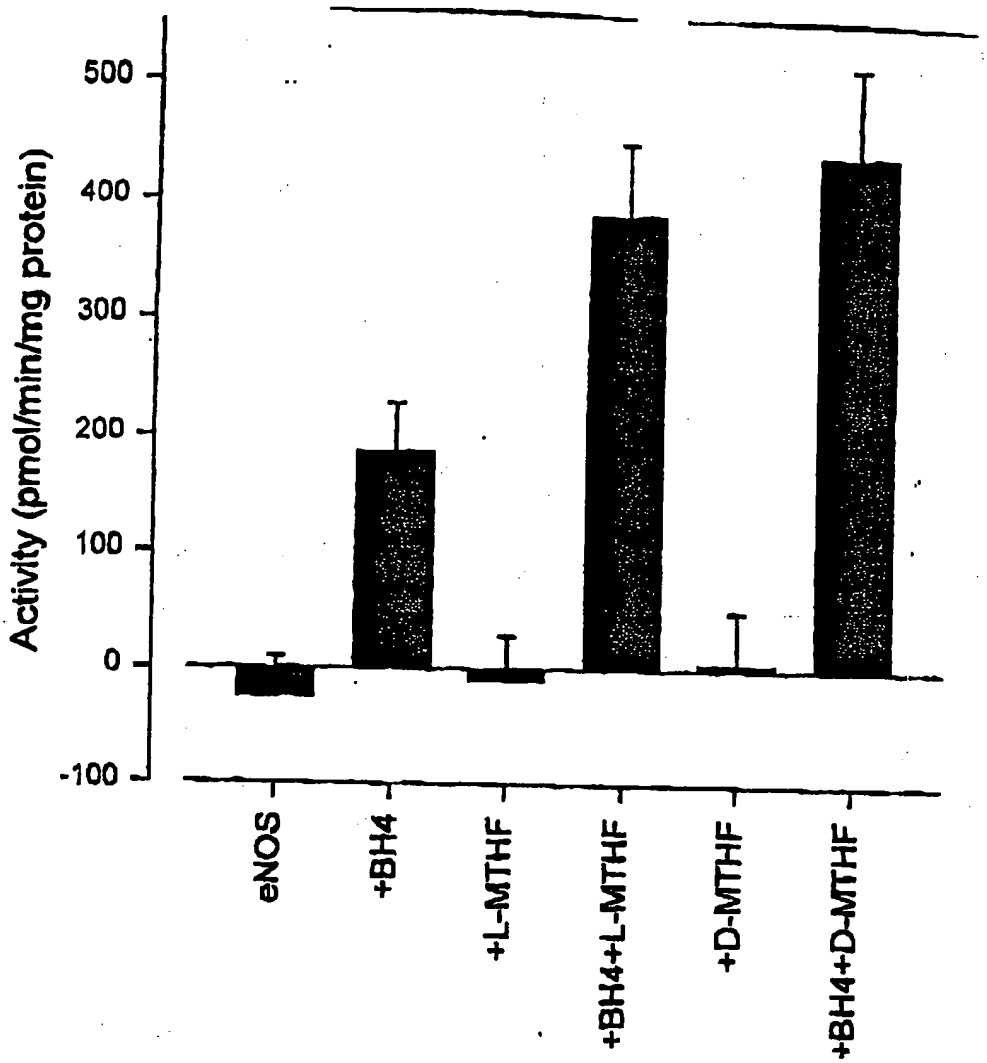

Figure 5

Effect of 5-methyl-(6S)- and –(6R)-tetrahydrofolic acid and tetrahydrobiopterin (BH_4) on NO production

Pterin-free eNOS produces no NO. Addition of BH_4 results in significant NO production. Both 5-methyl-(6S)- and –(6R)-tetrahydrofolic acid cause a further increase in NO-production by pterin-repleted eNOS, whereas 5-methyltetrahydrofolic acid has no effect on pterin-free eNOS.

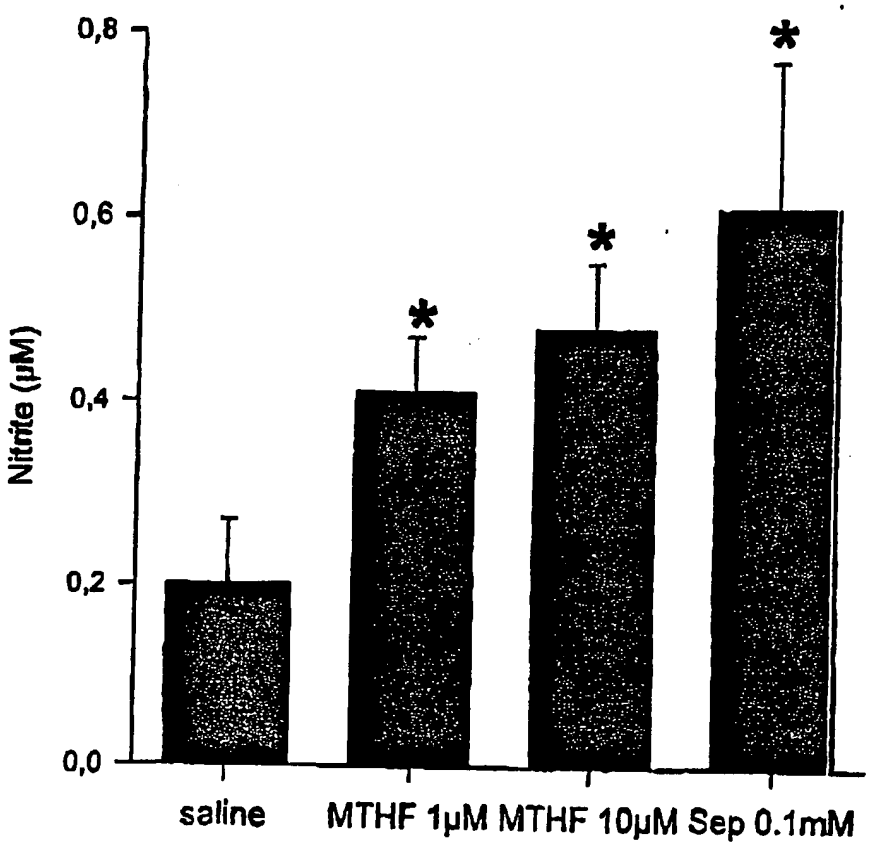

Figure 6

Effect of 5-methyl-(6S)-tetrahydrofolic acid and sepiapterin on acetylcholine-stimulated nitrite production by endothelial cells

Preincubation with 5-methyltetrahydrofolic acid (1 and 10 µM) o sepiapterin (100 µM) significantly enhances acetylcholine-induced nitrite production from endothelial cells. *p<0.05 vs. saline

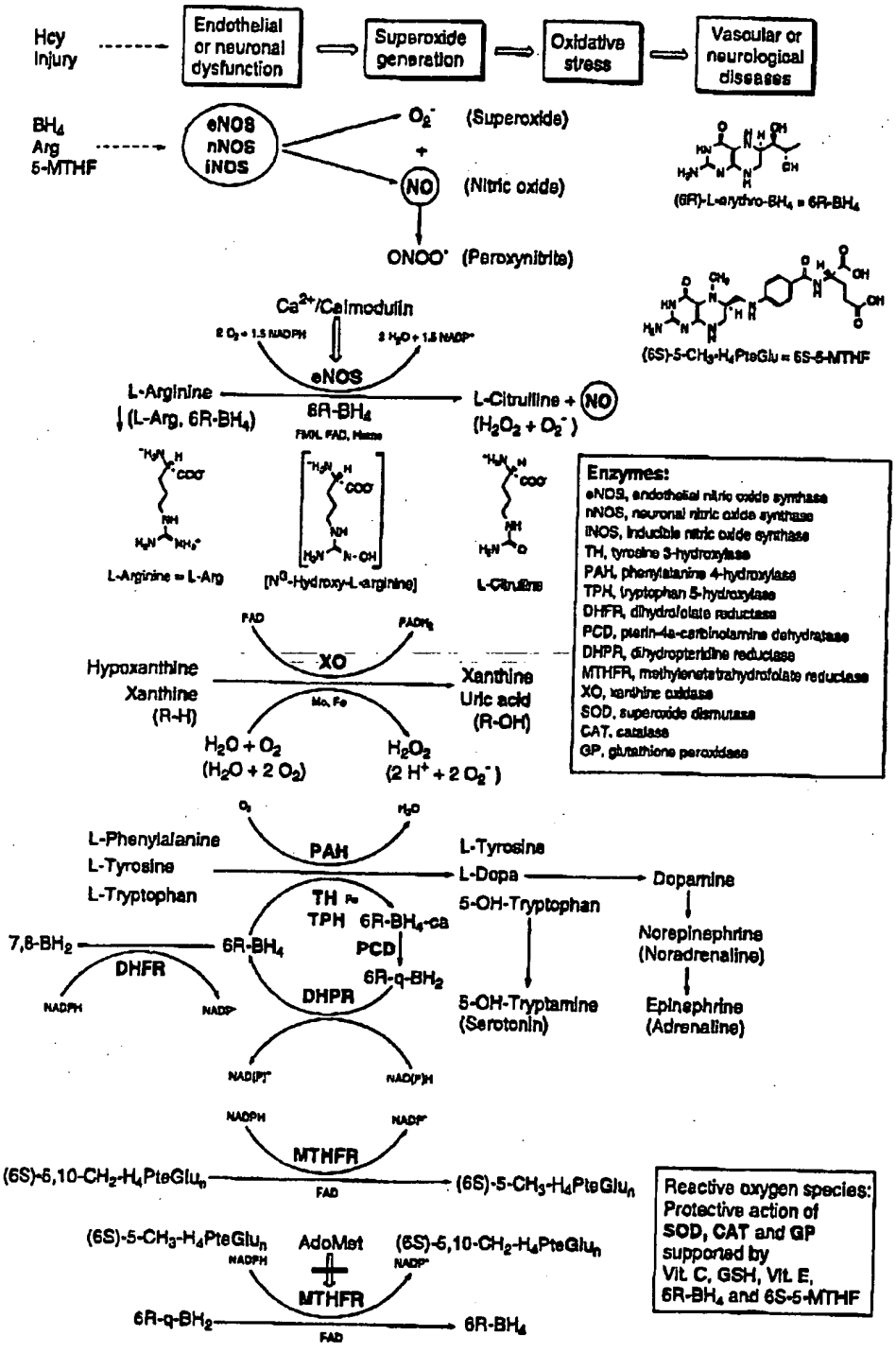

Figure 7

Overview of biochemical functions of folates and tetrahydrobiopterin regarding nitric oxide and oxidative stress

PHARMACEUTICAL PREPARATION CONTAINING AT LEAST FOLIC ACID OR A FOLATE AND TETRAHYDROBIOPTERIN (BH4) OR DERIVATIVES THEREOF USED FOR TREATING OR PREVENTING CARDIOVASCULAR OR NEUROLOGICAL DISORDERS BY MODULATING OF THE ACTIVITY OF NITRIC OXIDE SYNTHASE (NOS)

[0001] The invention relates to the use of at least folic acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof for treating or preventing cardiovascular or neurological disorders by modulation of the activity of nitric oxide synthase (NOS). The present invention also relates to the use of at least folic acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof for the production of a pharmaceutical preparation suitable for influencing the nitric oxide (NO) level, particularly by modulation of the activity of nitric oxide synthase (NOS) by reducing superoxide (O_2) production and enhancing nitric oxide (NO) synthesis. This effect occurs in the absence of any negative changes in other risk factors, e.g. lipids, blood pressure and homocysteine. Clinical areas of application include all anomalies of the nitric oxide level, particularly the prevention and treatment of cardiovascular and of neurological disorders. The present invention also relates to pharmaceutical preparations comprising at least one compound selected from the group consisting of 5-formyl-(6 S)-tetrahydrofolic acid, 5-methyl-(6 S)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methanyl-(6R)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5-formimino-(6 S)-tetrahydrofolic acid or (6 S)-tetrahydrofolic acid, together with tetrahydrobiopterin (BH_4) or pharmaceutically compatible salts thereof and with pharmaceutically compatible active and adjuvant substances, such as arginine for influencing the nitric oxide (NO) level.

[0002] Within this text the term a folate or a derivative thereof, if not explicitly defined otherwise, always refers to the natural and unnatural stereoisomeric form of each substance, pharmaceutically compatible salts thereof and any mixtures of the isomers and the salts. As drugs, tetrahydrofolates have predominantly been used hitherto as the calcium salt of 5-formyl-5,6,7,8-tetrahydrofolic acid (leucovorin) or of 5-methyl-5,6,7,8-tetrahydrofolic acid (MTHF) for the treatment of megaloblastic folic acid deficiency anemia, as an antidote for increasing the compatibility of folio acid antagonists, particularly of aminopterin and methotrexate in cancer chemotherapy ("antifolate rescue"), for increasing the therapeutic effect of fluorinated pyrimidines and for the treatment of autoimmune diseases such as psoriasis and rheumatoid arthritis, for increasing the compatibility of certain antiparasitic agents, for instance trimethoprim-sulfamethoxazole, and for decreasing the toxicity of dideazatetra-hydrofolates in chemotherapy and for influencing the homocysteine level, particularly for assisting the remethylation of homocysteine.

[0003] The term tetrahydrobiopterin (BH_4) or a derivative thereof, if not explicitly defined otherwise, always refers to all natural and unnatural stereoisomeric forms of tetrahydrobiopterin, pharmaceutically compatible salts thereof and any mixtures of the isomers and the salts. The term tetrahydrobiopterin also includes any precursors of tetrahydrobiopterin, especially 7,8-dihydrobiopterin. (6R)-tetrahydrobiopterin is a naturally occuring cofactor of the aromatic amino acid hydroxylases and is involved in the synthesis of the three common aromatic amino acids tyrosine, phenylalanine, tryptophan and the neurotransmitters dopamine and serotonin. It is also essential for nitric oxide synthase catalysed oxidation of L-arginine to L-citrullin and nitric oxide. Tetrahydrobiopterin is involved in many other biochemical functions, many of which have been just recently discovered.

[0004] The term arginine, if not explicitly defined otherwise, always refers to the natural and unnatural stereoisomeric form of arginine. L-arginine, a natural amino acid, is the precursor of endogenous nitric oxide (NO), which is a ubiquitous and potent vasodilator acting via the intracellular second-messenger cGMP. In healthy humans, L-arginine induces peripheral vasodilation and inhibits platelet aggregation due to an increased NO production. Both an excess and a lack of production of NO have been linked to pathological conditions, including cardiovascular disorders, septic shock, inflammation and infection, and brain damage in stroke and neurological disorders. The term nitric oxide synthase (NOS), if not explicitly defined otherwise, always refers to all isoforms endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS).

[0005] Nitric oxide (NO) has been identified as a mediator of atherosclerosis. Therefore it is a therapeutic target in cardiovascular prevention trials. It also plays an important role in neurological disorders. Biological effects of nitric oxide (NO) are not limited to vascular relaxation, but are also important in the respiratory, urogenital and gastrointestinal system, central and peripheral nervous system, neuroendocrine and endocrine systems, and nonspecific immunity.

[0006] Nitric oxide (NO) and superoxide (O_2) are cytotoxins on their own, yet it has been demonstrated that the two relatively unreactive radicals can rapidly combine ($k=3.7\times10^{7}\ M^{-1}\ s^{-1}$) under physiological conditions to the strong oxidizing agent peroxynitrite ($ONOO^-$). This reaction is about 3 times faster than the detoxifying catabolism of superoxide by superoxide dismutase (SOD). It is believed that the formation of peroxynitrite is an important factor in the oxidative damage associated with ischemia/reperfusion. A variety of pathologies are associated with the formation of peroxynitrite. Peroxynitrite is invariably formed in larger amounts when more NO is produced, and/or when an elevated level of superoxide prevails. In this regard, pathologies such as diabetes, atherosclerosis, and ischemia-reperfusion injury, are associated with oxidative stress characterized by an elevated level of superoxide that can lead to increased peroxynitrite formation. Also when glutathione detoxification mechanism against peroxynitrite is impaired critical concentrations of peroxinitrite may occur. Recent evidence also suggests multiple sclerosis and Alzheimer's disease are associated with peroxynitrite formation. In addition, peroxynitrite has also been implicated during sepsis and adult respiratory distress syndrome. Ischemia and reperfusion are accompanied by an increase in superoxide due to the activation of xanthine oxidase and NAPDH oxidase, respectively. Thus, peroxynitrite is likely to be implicated in a number of pathologies in which an imbalance of NO and superoxide occurs.

[0007] Several factors can contribute to reduced bioavailability of NO, ranging from impaired production to

increased degradation, depending on the risk factors involved. NO is synthesized by dimers of the 130 kD enzyme endothelial NO synthase in a reaction where arginine is oxidized to NO and citrulline. It has been shown that eNOS produces superoxide radicals as well as NO. Under physiological conditions, NOS predominantly produces NO, controlled by the regulatory co-enzyme calmodulin, the substrate arginine and the cofactor tetrahydrobiopterin (BH_4). Under pathophysiological conditions, such as dyslipidemia, production shifts from NO to superoxide. Clinical studies have shown impaired NO bio-availability in patients with (risk factors for) atherosclerosis. Evidence has accumulated showing that increased production of superoxide and increased degradation of NO by superoxide, rather than impaired formation of NO is the predominant cause of impaired NO bioavailability in early atherosclerosis. These observations indicate that atherogenesis is linked to a pathological imbalance between NO and superoxide, rather than reduced NO production per se.

[0008] The level of superoxide can be lowered by substances showing a relevant scavenging capacity for superoxide radicals. Measurements revealed that arginine does not react with superoxide. However, both arginine and tetrahydrobiopterin (BH_4) are required to minimize or abolish superoxide formation by NOS. Tetrahydrobiopterin (BH_4) shows a reaction rate with superoxide which is roughly 2 fold smaller than that of the potent antioxidant ascorbic acid and for folic acid, folates or derivatives thereof (as an example 5-methyl-(6 S)- and (6R)-tetrahydrofolic acid have been measured), the reaction rates are about 20 times slower than that of ascorbic acid. Beside of its tenfold lower scavenging capacity folic acid, folates or derivatives thereof are different from tetrahydrobiopterin (BH_4) or derivatives thereof in that achievable plasma concentrations are far lower. Upon standard oral suppletion of folic acid (5 mg p.o.) systemic plasma concentrations of 5-methyltetrahydrofolic acid up to ca. 150 nM are achieved whereas upon intra-arterial infusion values of 250 nM were reached. Both these interventions have been shown to result in an improvement in NO-availability in hypercholesterolemic patients. Still these levels of folic acid, folates or derivatives thereof remain orders of magnitude below those of ascorbic acid (concentrations up to 50 μM).

[0009] Despite of the situation that it has been known that "a scavenging effect of BH_4 had been remarked"[Vasquez-Vivar, J. et al., Proc. Natl. Acad. Sci. U.S.A., 1998, 95, 9220-9225], "exogenous BH_4 is capable of restoring impaired NO activity in prebypertensive rats"[Cosentino, F. et al., J. Clin. Invest., 1998, 101, 1530-1537], "exogenous BH_4 is capable of restoring impaired NO activity in hypercholesterolemia patients"[Stroes, E. at al., J. Clin. Invest., 1997, 99, 41-46], "exogenous BH_4 is capable of restoring impaired NO activity in diabetic patients"[Pieper, G. M., J. Cardiovasc. Pharmacol., 1997, 29, 8-15], "folate therapy improves NO activity during hypercholesterolemia in vivou"[Woo, K. S. at al., Circulation, 1998, 97, I-165-166] and [Verhaar, M. C. et al., Circulation, 1998: 97 (3), 237-241), "folic acid and its active form 5-MTHF restore impaired NO bioavailability in dyslipidemic conditions" [Wilmink, H. et al., Arteriosclerosis Thromb Vasc Biol, 2000; 20 (1), 185-8] and [Verhaar, M. C. et al., Circulation, 1998; 97 (3), 237-241], "clinical studies have revealed that the impairment of endogenous vasodilator function observed with hypercholesterolemia is reversible by administration of L-arginine"[Creager, M. A. et al., Clin Invest. 1992, 90, 1248-1253] and "Folic acid supplementation improves arterial endothelial function in adults with realtive hyperhomocysteinemia"[Woo, K. S. et al., J. Am. College of Cardiology, 1999, 34 (7), 2002-2006] the use of at least folic acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof together with pharmaceutically compatible active and adjuvant substances, such as arginine for the production of a pharmaceutical preparation suitable for influencing the nitric oxide (NO) level has neither been proposed nor described hitherto.

[0010] This is probably due to the situation that it has been postulated that "MTHF had no direct effect on in vitro NO production by eNOS"[Verhear, M. C. et al., Circulation, 1998; 97 (3), 237-241].

[0011] It has been found that the use of pharmaceutical preparations containing at least folic acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof influences the nitric oxide (NO) level, and in particular affects the enzymatic activity of nitric oxide synthase (NOS) by reducing superoxide production and enhancing nitric oxide (NO) synthesis. This effect occurs in absence of negative changes in other risk factors, e.g. lipids, blood pressure and homocysteine.

[0012] Especially surprising is this effect as in pterin-free eNOS folic acid, a folate or a derivative thereof does not affect the enzymatic activity of nitric oxide synthase (NOS), neither with regard to NO, nor to superoxide production, whereas in partially pterin-repleted eNOS folic acid, a folate or a derivative thereof have the claimed strong effect on the activity of the enzyme; i.e. they enhance NO production concomitant with a decreased production of superoxide. The beneficial vascular effect of folic acid or a folate together with at least tetrahydrobiopterin (BH_4) or derivatives thereof cannot be attributed solely to direct scavenging of superoxide.

[0013] Folic acid, a folate or a derivative thereof refers to folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof or a combination of two or more thereof. Especially means folic acid, a folate or a derivative thereof folic acid, dihydrofolate, tetrahydrofolate, 5-methyltetrahydrofolate, 5,10-methylenetetrahydrofolate, 5,10-methenyltetrahydrofolate, 5,10 -formiminotetrahydrofolate, 5-formyltetrahydrofolate (leucovorin), 10-formyltetrahydrofolate 10-methyltetrahydrofolate, pharmaceutically compatible salts thereof, or a combination of two or more thereof.

[0014] Reduced folates can be converted into one another according to the well known folate metabolism. 5-methyltetrahydrofolic acid and the pharmaceutically compatible salts thereof are preferably used, however, since 5-methyltetrahydrofolic acid is directly involved together with tetrahydrobiopterin in such functions as the biosynthesis of dopamine, norepinaphrine and serotinine by the hydroxylation of phenylalanine and the regeneration of BH_4 by the reduction of the quinonoid 7,8-dihydrobiopterin to tetrahydrobiopterin.

This applies in particular when there is an existing methylenetetrahydrofolate reductase deficiency, wherein this deficiency implies disorders such as restricted functionality or lack of activity, for example. The existence of thermolabile methylenetetrahydrofolate reductase should be mentioned here as the most frequent example of a methylenetetrahydrofolate reductase deficiency. Under these circumstances, especially 5-methyltetrahydrofolic acid is only available in a limited amount.

[0015] Within all folates or a derivatives thereof both the natural and the unnatural diastereoisomers, pharmaceutically compatible salts thereof and any mixtures of the isomers and the salts, but especially the natural diastersoisomeric forms such as 5-methyl-(6 S)-tetrahydrofolic acid are applicable.

[0016] Tetrahydrobiopterin (BH_4) refers to all the natural and the unnatural forms of tetrahydrobiopterin, pharmaceutically compatible salts thereof and any mixtures of the isomers and the salts, but especially the natural diastereoisomeric form (6R)-L-erythro-tetrahydrobiopterin is applicable.

[0017] Arginine refers to the both the natural and unnatural isomeric form of arginine, pharmaceutically compatible salts thereof and any mixtures of the isomers and the salts, but especially the natural isomeric form L-arginine is applicable.

[0018] For an overview of the biochemical functions of folates and tetrahydrobiopterin regarding nitric oxide and oxidative stress see **FIG. 7**.

[0019] Pharmaceutically compatible salts should be both pharmacologically and pharmaceutically compatible. Pharmacologically and pharmaceutically compatible salts such as these may be alkali or alkaline earth metal salts, preferably sodium, potassium, magnesium or calcium salts.

[0020] The expression "pharmaceutical preparations" refers to enteral (e.g. oral, sublingual or rectal), parenteral or topical (e.g. transdermal) forms, Organic or inorganic substances which do not react with the active ingredient can be used as supports, e.g. water, oil, benzyl alcohol, polyethylene glycol, glycerol triacetate or other fatty acid glycerides, gelatine, lecithin, cyclodextrin, carbohydrates such as lactobiose or starch, magnesium stearate, talc or cellulose. Tablets, dragees, capsules powders, syrup concentrates or drops are preferred for oral application, suppositories are preferred for rectal application, and water- or oil-based solutions or lyophilisates are preferably used for parenteral application.

[0021] Suspensions, emulsions or implants can also be used, and patches or creams can be used for topical application.

[0022] Pharmaceutical preparations for parenteral application comprise sterile aqueous and non-aqueous injection solutions of the pharmaceutically-active compounds, which are preferably isotonic with the blood of the recipient.

[0023] These preparations may comprise stabilizers, additives for the controlled release of the pharmaceutically-active compounds, antioxidants, such as ascorbic acid, reduced glutathione or N-acetyl-cysteine, buffers, bacteriostatic agents and adjuvant substances for obtaining an isotonic solution. Aqueous and non-aqueous sterile suspensions may contain suspension additives and thickeners. The pharmaceutical preparation may exist as a single dose- or as a multiple-dose container, as sealed ampoules for example, and may be stored as a freeze-dried (lyophilized) product and prepared for use if need be with a sterile liquid, for example water or salt solution. Sterile powders, granules or tablets can be used in the same manner. All the pharmaceutical preparations may additionally contain active compounds which act separately or synergistically, Arginine should be mentioned here, which has a synergistic effect in this application. In this respect, arginine can be used in a dose between 1 mg and 1 g, preferably between 1 mg and 100 mg per day, for a normal dosage application, and can be used in a dose between 10 mg and 1 g per day for a high dosage application.

[0024] The pharmaceutical preparation contains between 0.001 mg and 1000 mg of folic acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof together with 1 mg to 10 g of arginine per dose. In prophylaxis, preparations are used which preferably contain between 5 μg and 1000 mg of the active ingredient per dose. In therapy, preparations are used which preferably contain between 0.1 mg and 200 mg of folic acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof together with 1 mg to 1 g of arginine per dose.

[0025] The dosage depends on the form of therapy, on the form of application of the pharmaceutical preparation, and on the age, weight, nutrition and condition of the patient. Treatment may be commenced with a low dosage below the optimum amount and this may be increased until the optimum effect is achieved. The dosages used in prophylaxis may preferably vary for folic acid or a folate and tetrahydrobiopterin (BH_4) between 5 μg and 1000 μg per day, particularly between 50 μg and 500 μg per day. Optimum dosages in therapy vary for folic acid or a folate and tetrahydrobiopterin (BH_4) between 0.1 mg and 100 mg per day, particularly between 0.5 mg and 5 mg per day. Application may be effected as a single administration or as a repeated dosage.

EXAMPLES TO ILLUSTRATE THE INVENTION

[0026] Chemicals

[0027] BH_4-free bovine eNOS was obtained through expression of eNOS in *E coli.* 5-methyl-(6 S)-tetrahydrofolic acid, and its stereoisomer, 5-methyl-(6R)-tetrahydrofolic acid were used in purities>99.8%. The spin trap, 5-diethoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) and all other used chemicals are commercially available.

[0028] Electron Paramagnetic Resonance Measurements (EPR)

[0029] The EPR spectra were recorded at 37° C. on a modified Bruker ESP 300. Spin trap experiments were performed with both HX/XO and eNOS. For HX/XO the solution contained 0.5 mM hypoxanthine, 12.5 mU/ml xanthine oxidase and 50 mM DEPMPO in phosphate buffer (pH 7.4). The eNOS assay contained 250 nM eNOS dimers (0.065 mg protein/ml), 0.5 mM NADPH, 10 μM L-arginine, 1 mM $CaCl_2$, 300 U/ml catmodulin and 50 mM DEPMPO in phosphate buffer (pH 7.4).

[0030] Determination of NO-production by eNOS

[0031] NOS activity was determined by quantifying the conversion of L-[2,3,4.5-^{3}H]arginine into L-[2,3,4,5-^{3}H]-citrulline. Briefly, 2 μg eNOS (BH_4-free or -repleted) was incubated during 5 min at 37° C. in 100 μl HEPES buffer

(pH 7.4) containing DTPA (0.1 mmol/l), $CaCl_2$ (0.2 mmol/l), calmodulin (20 μg/ml), NADPH (0.5 mmol/l), FMN (1 μmol/l), FAD (1 μmol/l), glutathione (100 μmol/l), BSA (200 μg/ml), L-arginine (100 μmol/l), and L-[2,3,4,5-^{3}H]-arginine (3.7 KBq). All measurements were performed in triplicate. After correction for nonspecific activity, eNOS activity was calculated from the percent conversion of [^{3}H]-arginine into [^{3}H]-citrulline and expressed as nmoles per mg protein per min.

[0032] Cell cultures

[0033] Microvascular endothelial bEND3 cells were cultured to confluence in 6-well culture plates for determination of nitrite or in 15-cm dishes for electron spin resonance experiments. After the cells reached confluence the medium was changed to M-199 (Sigma chern), supplemented with 0.1% BSA, 5mM L-glutamine, antibiotics and 5-methyltetrahydrofolic acid (0, 1, 10 μM) or sepiapterin (100 μM), respectively, for 24 hours.

[0034] Determination of NO production by endothelial calls

[0035] The NO production by endothelial cells was assessed by quantification of the nitrite content in the supernatant with a commercially available fluorimetric kit (Cayman Chemicals). Acetylcholine-induced NO production is presented as the difference between stimulated minus the unstimulated nitrite content.

[0036] Statistical analysis

[0037] Changes in NO-production were tested with an unpaired t-test. Changes in radical adduct formation by tetrahydrobiopterin (**FIG. 3**) or 5-methyl-(6 S)-tetrahydrofolic acid (**FIG. 4**) were tested with analysis of variance. If variance ratios reached statistical significance, differences between the means were analyzed with the Student-Newman-Keuls test for $p<0.05$.

[0038] 5-Methyltetrahydrofolic acid direct Superoxide Scavenging

[0039] XO Activity

[0040] Assessment of urate levels (assessed with a uricase-hydrogen peroxide assay) is a standard method to determine enzymatic activity of XO. However, reductive substance, like vitamin C or NUHF, are known to interfere with the urate determination. Basal urate levels after 60 minutes of incubation (1, 2, 5 mU/ml XO, 0.5 mM hypoxanthine in phosphate buffer, pH 7.4, 37° C.) were 128±20 μM. Addition of 50 μM 5-methyl-(6 S)-tetrahydrofolic acid at the beginning of the incubation period resulted in a significantly lower urate level of 48.7±1.7 μM. Addition of 50 μM 5-methyl-(6 S)-tetrahydrofolic acid at the end of the incubation period (just prior to urate assessment) resulted in a similar urate level (49.9±1.9 μM). These data show that 5-methyl-(6 S)-tetrahydrofolic acid interferes with the quantification of urate, rather than the urate production itself and that 5-methyltetrahydrofolic acid does not affect the rate of urate production by XO.

[0041] Determination of Superoxide Trapping Rates by Competitive Superoxide Trapping

[0042] The trapping rates for superoxide were determined at 37° C. by comparing the trapping efficiency of L, arginine, tetrahydrobiopterin and 5-methyl-(6 S)- and -(6R)-tetrahydrofolic acid with the known trapping efficiency of the spin trap DEPMPO (competitive superoxide trapping [CST]). Using HX/XO as superoxide generating system, the presence of other compound (like L-arginine, tetrahydrobiopterin, 5-methyftetrahydrofolic acid or ascorbic acid) will result in less generation of DEPMPO spin adducts. Reaction channels other than with DEPMPO, L-arginine, tetrahydrobiopterin, 5-methyltetrahydrofolic acid or ascorbic acid can be neglected as the adduct yield does not increase further if DEPMPO concentrations higher than 50 mM are used. The time curves of the EPR intensity in the HX/XO system were described by single exponentials with a time constant, t=10±0.5 min (**FIG. 1**). Plots of the steady state limits as a function of 5-methyl-(6 S)- and -(6R)-tetrahydrofolic acid concentration in the HX/XO system are given in **FIG. 2** (solid circles). Both isomers show the same linear concentration dependence.

[0043] The reaction rates with superoxide are given by k for the tested substance and k_d for the reference compound DEPMPO respectively. A reference value of $k_d=80\ (Ms)^{-1}$ has been shown to be reliable. Based thereon the following values have been determined at pH 7.4 and 37° C.

$k_{ascorbic\ acid}/k_d=4400$ $k_{ascorbic\ acid}=3.5\times10^5\ (Ms)^{-1}$ [literature value $k_{ascorbic\ acid}=2.7\times10^5\ (Ms)^{-1}$]

$k_{BH4}/k_d=1200$ $k_{BH4}=1.5\times10^5\ (Ms)^{-1}$

$k_{L\text{-}MTHF}/k_d=150$ $k_{L\text{-}MTHF}=1.2\times10^4\ (Ms)^{-1}$

$k_{D\text{-}MTHF}/k_d=150$ $k_{D\text{-}MTHF}=1.2\times10^4\ (Ms)^{-1}$

$k_{arginine}/k_d=<10^{-2}$ $k_{arginine}$=neglibile

[0044] The presence of L-arginine did not affect the formation of DEPMPO adducts, even at high concentrations (up to 100 mM). Therefore, L-arginine has no significant scavenging capacity for superoxide.

[0045] BH_4 is an about 2 times less potent scavenger than ascorbic acid, whereas both isomers of 5-methyltetrahydrofolic acid are in this regard about 20 times less potent than ascorbic acid. In addition the usual plasma concentration for tetrahydrobiopterin and folates are in the low nanomolar range. Upon oral supplementation, the level of folic acid may be raised to micromolar range, which is still below the vitamin C levels of 30-50 micromolar observed in vivo. Therefore due to the low scavenging potency and low plasma concentration of BH_4 and folates their direct superoxide scavenging capacity is not relevant in vivo, where antioxidant mechanisms like vitamin C or superoxide dismutase (SOD) have far higher capacity for removal of superoxide. Instead, folates exerts their beneficial effects together with tetrahydrobiopterin through modulation of the enzymatic activity of NOS.

[0046] 5-Methyltetrahydrofolic acid-Pterin-Free eNOS

[0047] Superoxide Production by Pterin-Free eNOS

[0048] To elucidate whether that 5-methyl-(6 S)-tetrahydrofolic acid improves NO bioavailability in vivo in hypercholesterolemic patients by a direct effect of 5-methyl-(6 S)-tetrahydrofolic acid on eNOS, competitive superoxide trapping (CST) experiments using eNOS as a superoxide generating system have been carried aout. The effect of 5-methyl-(6 S)- and -(6R)-tetrahydrofolic acid on the pterin-free eNOS (**FIG. 3**, solid circles) coincides with the data from the HX/XO experiments (c.f. **FIG. 2**). It demonstrates that for pterin-free eNOS impaired formation of spin adducts

can be fully accounted for by the capacity of 5-methyl-(6 S)- and -(6R)-tetrahydrofolic acid to scavenge superoxide in a bimolecular scavenging reaction. In particulars the presence of 5-methyl-(8 S)- or -(6R)-tetrahydrofolic acid does not affect the rate of superoxide production by pterin-free eNOS.

[0049] In pterin-free eNOS, folic acid, a folate or a derivative thereof significantly reduce the formation of DEPMPO superoxide adducts. The degree of reduction in superoxide activity by folic acid, a folate or a derivative thereof is equivalent to that observed in the HX/XO system. It shows that for pterin-free eNOS folic acid, a folate or a derivative thereof exerts its effects through pure scavenging only, without interfering with enzymatic activity. Combining the results for NO and superoxide, pterin-free eNOS is seen to be completely oblivious to the presence of folic acid, a folate or a derivative thereof.

[0050] 5-meothyltetrahydrofolic acid-Pterin-Repleted eNOS

[0051] Superoxide Production by eNOS.—Pterin-Repleted Case

[0052] As far as pterin-repleted eNOS is concerned (FIG. 3, open circles), addition of 5-methyl-(6 S)- or -(6R)-tetrahydrofolic acid-results in-a very strong reduction in the rate of DEPMPO adduct formation (as manifests itself from a much steeper slope). This reduction by far exceeds the reduction observed in pterin-free eNOS. This observation cannot be fully explained by the capacity of 5-methyl-(6 S)- and -(6R)-tetrahydrofolic acid to scavenge superoxide. Therefore the presence of 5-methyltetrahydrofolic acid reduces the superoxide production by eNOS in a concentration dependent way with both stereoisomers of 5-methyltetrahydrofolic acid having the same potency. To evaluate whether 5-methyltetrahydrofolic acid only affects eNOS after preincubation with BH_4, increasing amounts of BH_4 have been added to pterin-free eNOS in presence and absence of 5-methyl-(6 S)-tetrahydrofolic acid 25 μM (FIG. 4). As expected, in the absence of 5-methyltetrahydrofolic acid, addition of BH_4 caused a dose-dependent decrease in superoxide production. In pterin-free eNOS, addition of 25 μM 5-methyl-(6 S)-tetrahydrofolic acid does not cause significant changes in radical adduct formation (FIG. 4). In contrast, addition of 5-methyltetrahydrofolic acid to partially repleted eNOS (still BH_4 deficient) causes a substantial reduction in the amount of superoxide adducts (FIG. 4).

[0053] NO-Production by eNOS

[0054] The NO production by pterin-free eNOS is located at the detection limit of the arginine-citrulline conversion assay (FIG. 5). Addition of 5-methyl-(6 S)- or -(6R)-tetrahydrofolic acid (100 μM final) to pterin-free eNOS has no significant effect on NO production (FIG. 5). In contrast, in pterin-repleted eNOS, two significant differences arise: first, a clear basal NO-production is observed (FIG. 5). Second, the addition of both 5-methyl-(6 S)- or -(6R)-tetrahydrofolic acid (100 μM final) causes a significant increase in NO production (FIG. 5; $p<0.05$ vs. BH_4 alone).

[0055] NO-Production by endothelial cells

[0056] Preincublation with 5-methyltetrahydrofolic acid did not affect nitrite release in unstimulated endothelial cells. Acetylcholine stimulation caused a significant increase in nitrite release (FIG. 6). Preincubation of endothelial cells with 5-methyl-(6 S)-tetrahydrofolic acid and sepiapterin resulted in a significant-further increase in acetylcholine-induced nitrite production (FIG. 6).

[0057] The pterin-repleted eNOS used In our studies shows substantial basal production of both NO as well as superoxide and therefore should be considered as BH_4 deficient, i.e. partially uncoupled. Under these conditions, addition of folic acid, a folate or a derivative thereof increases NO production.Both diastereoisomeric forms of the folates and derivatives thereof have the same effect. At the same time, the formation of DEPMPO superoxide adducts is strongly reduced by both folic acid, a folate or a derivative thereof. The reduction of adduct formation caused by folic acid, a folate or a derivative thereof by far exceeds that observed in the HX/XO system. This shows that the major impact of folic acid, a folate or a derivative thereof must be a direct interference with the enzymatic superoxide production by the pterin-repleted eNOS. Again, both diastereoisomeric forms of the folates have comparable effects. Combining the results, for NO and superoxide, the enzymatic activity of pterin-repleted eNOS is highly sensitive to the presence of folic acid, a folate or a derivative thereof. The overall effect is a substantial shift from superoxide production towards NO production. From FIG. 2, we estimate that superoxide production by eNOS is reduced by a factor of 2 at a concentration of [5-methyltetrahydrofolic acid]=50 μM, i.e. ca. 200 5-methyltetrahydrofolic acid molecules per eNOS dimer. Similar molecular ratios of 5-methyltetrahydrofolic acid vs. eNOS can be achieved in vivo upon oral supplemeatation with folic acid, a tolate or a derivative thereof.

[0058] 5-methyltetrahydrofolic acid-Pterin Interaction

[0059] It has been shown that folic acid, a folate or a derivative thereof, requires BH_4 before it can affect the enzymatic activity of eNOS. Folic acid, a folate or a derivative thereof supports the action as a cofactor of BH_4.

[0060] Moreover, therapy with folic acid, a folate or a derivative thereof did not show any effect on biopterin levels in vivo. Therefore folic acid, a folate or a derivative thereof exerts its effect via enhanced binding of BH_4 to eNOS,

[0061] Folic acid, a folate or a derivative thereof act as facilitator of the oxidation of BH_4 to the BH_4-radical.

[0062] 5-methyltetrahydrofolic acid-endothelial cells

[0063] It has been shown that the effects of folic acid, a folate or a derivative thereof on endogenous eNOS in endothelial cells are compatible with the findings on the recombinant enzyme. In particular, it has been shown an enhanced T40 status in cultured endothelial cells upon 5-methyltetrahydrofolic acid suppletion.

[0064] The decreased superoxide production and enhanced NO synthesis by the nitric oxide synthase (NOS) following the application of folic acid, a folate or a derivative thereof provides a plausible explanation for the increased NO bio-availability in humans upon 5-methyltetrahydrofolic acid suppletion during dyslipidaemia.

EXAMPLE 1

[0065] A tablet containing 50 mg 5-formyl-(6 S)-tetrahydrofolic acid and 50 mg (6R)-tetrahydrobiopterin (BH_4)

[0066] A mixture of 665 g of the pentahydrate of the calcium salt of 5-formyl-(6 S)-tetrahydrofolic acid (corresponding to 500 g 5-formyl-(6 S)-tetrahydrofolic acid), 645 g (6R)-tetrahydrobiopterin dihydrochloride (corresponding to 500 g (6R)-tetrahydrobiopterin), 4 kg lactose, 1.2 kg starch, 0.2 kg talc and 0.1 kg magnesium stearate is pressed to form tablets, so that each tablet contains 50 mg 5-formyl-(6 S)-tetrahydrofolic acid and 50 mg (6R)-tetrahydrobiopterin (BH_4).

[0067] The tablet can be coated as a film tablet or can be ground and used in capsule form.

EXAMPLE 2

[0068] A suppository containing 500 mg 5-methyl-(6 S)-tetrahydrofolic acid and 500 mg (6R)-tetrahydrobiopterin (BH_4)

[0069] A mixture of 632 g of the pentahydrate of the calcium salt of 5-methyl-(6 S)-tetrahydrofolic acid (corresponding to 500 g 5-methyl-(6 S)-tetrahydrofolic acid), 645 g (6R)-tetrahydrobiopterin dihydrochloride (corresponding to 500 g (6R)-tetrahydrobiopterin), 50 g hydroxy-propylcellulose and 2 kg of semisynthetic glycerides is melted to form suppositories, so that each suppository contains 500 mg 5-methyl-(6 S)-tetrahydrofolic acid and 500 mg (6R)-tetrahydrobiopterin (BH_4).

EXAMPLE 3

[0070] An injection solution containing 5 mg 5-methyl-(6 S)-tetrahydrofolic acid, 1 mg (6R)-tetrahydrobiopterin (BH_4) and 5 mg L-arginine

[0071] 5.0 g 5-methyl-(6 S)-tetrahydrofolic acid, 1.0 g (6R)-tetrahydrobiopterin (BH_4), 5.0 g L-arginine, 10 g glutathione, 30 g citric acid, 160 g mannitol, 1 g methyl-p-hydroxybenzoic acid, 17.7 g sodium hydroxide (or the requisite amount in order to obtain a pH of the solution of 7.3 to 7.8) is dissolved in 3 liters of water for injection and introduced into ampoules, so that each ampoule contains 5 mg 5-methyl-(6 S)-tetrahydrofolic acid, 1 mg (6R)-tetrahydrobiopterin (BH_4) and 5 mg L-arginine.

EXAMPLE 4

[0072] An injectable lyophilisate containing 1 mg tetrahydrofolic acid and 1 mg tetrahydrobiopterin (BH_4)

[0073] A solution of 1.05 g of the sodium salt of tetrahydrofolic acid (corresponding to 1.0 g tetrahydrofolic acid) and 1.40 g (6R)-tetrahydrobiopterin sulfate (corresponding to 1.0 g (6R)-tetrahydrobiopterin) in 1000 ml double-distilled water is introduced via sterile filtration into ampoules and lyophilised, so that each ampoule contains 1 mg tetrahydrofolic acid and 1 mg tetrahydrobiopterin (BH_4).

[0074] Tetrahydrofolic acid is very sensitive to oxygen and stringently oxygen-free conditions therefore have to be employed. The use of an antioxidant such as ascorbic acid may be necessary.

EXAMPLE 5

[0075] An injectable lyophilisate containing 20 mg 5,10-methylene-(6R)-tetrahydrofolic acid and 50 mg (6R)-tetrahydrobiopterin (BH_4)

[0076] A solution of of the .beta.-hydroxypropyl-cyclodextrin inclusion compound of the sodium salt of 5,10-methylene-(6R)-tetrahydrofolic acid containing 10 g 5,10-methylene-(6R)-tetrahydrofolic acid and 50 g (6R)-tetrahydrobiopterin (BH_4) in 2000 ml of double-distilled water is introduced via sterile filtration into ampoules, so that each ampoule contains 20 mg 5,10-methylene-(6R)-tetrahydrofolic acid and 50 mg (6R)-tetrahydrobiopterin (BH_4).

[0077] The same precautionary measures apply to 5,10-methylene-tetrahydrofolic acid as for tetrahydrofolic acid (preceding Example).

EXAMPLE 6

[0078] A tablet containing 4 mg 5-formyl-(6 S)-tetrahydrofolic acid and 10 mg (6R)-tetrahydrobiopterin (BH4)

[0079] A mixture of 53.2 g of the pentahydrate of the calcium salt of 5-formyl-(6 S)-tetrahydrofolic acid (corresponding to 40 g 5-formyl-(6 S)-tetrahydrofolic acid), 100 g (6R)-tetrahydrobiopterin (BH_4), 4 kg lactose, 1.2 kg starch, 0.2 kg talc and 0.1 kg magnesium stearate is pressed to form tablets, so that each table contains 4 mg 5-formyl-(6 S)-tetrahydrofolic acid and 10 mg (6R)-tetrahydrobiopterin (BH_4).

[0080] The tablet can be coated as a film tablet or can be ground and used in capsule form.

EXAMPLE 7

[0081] An injectable lyophilisate containing 10 μg 6-methyl-(6 S)-tetrahydrofolic acid and 10 μg (6R)-tetrahydrobiopterin (BH_4)

[0082] A solution of 10 mg of the sodium salt of 5-methyl-(6 S)-tetrahydrofolic acid and 10 mg (6R)-tetrahydrobiopterin (BH_4) in 1000 ml of double-distilled water is introduced, via sterile filtration under an inert gas, into ampoules and lyophilised, so that each ampoule contains 10 μg 5-methyl-(6 S)-tetrahydrofolic acid and 10 μg (6R)-tetrahydrobiopterin (BH_4). Tetrahydrofolic acid is very sensitive to oxygen, and stringently oxygen-free conditions therefore have to be employed. The use of an antioxidant such as ascorbic acid may be necessary.

EXAMPLE 8

[0083] A tablet containing 15 mg 5-methyl-(6 S)-tetrahydrofolic acid and 5 mg (6R)-tetrahydrobiopterin (BH_4)

[0084] A mixture of 19.18 g of the pentahydrate of the calcium salt of 5-methyl-(6 S)-tetrahydrofolic acid (corresponding to 15 g 5-methyl-(6 S)-tetrahydrofolic acid), 5 g (6R)-tetrahydrobiopterin (BH_4), 120 g lactose, 21.5 g maize starch, 7.08 g acetylcellulose, 2.28 g diethyl phthalate, 0.64 g silicone HK-15 and 2 g magnesium stearate is pressed to form tablets, so that each tablet contains 15 mg 5methyl-(6 S)-tetrahydrofolic acid and 5 mg (6R)-tetrahydrobiopterin (BH_4).

[0085] The tablet can be coated as a film tablet or can be ground and used in capsule form.

EXAMPLE 9

[0086] Tablets containing 10 mg 5-methyl-(6 S)-tetrahydrofolic acid and 10 mg (6R)-tetrahydrobiopterin (BH_4)

[0087] In an analogous manner to that described in Example 8, tablets containing 10 mg 5-methyl-(6 S)-tetrahydrofolic acid and 10 mg (6R)-tetrahydrobiopterin (BH_4) are produced using maize starch, lactose, magnesium stearate, polyethylene glycol 6000, polymethacrylate, polysorbitol 80, dimethylpolysiloxane, sodium hydroxide and talc.

EXAMPLE 10

[0088] A combination preparation comprising 5-methyltetrahydrofolic acid, tetrahydrobiopterin (BH_4) and arginine

[0089] A film tablet which contains the following constituents is formulated for preparations for oral application:

25 mg	5-methyltetrahydrofolic acid
25 mg	tetrahydrobiopterin (BH_4)
250 mg	arginine
	pharmaceutically compatible adjuvant substances

[0090] The tablet can be coated as a film tablet or can be ground and used in capsule form.

[0091] This combination preparation may also be formulated as a solution, e.g. for parenteral application.

[0092] The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples. Also, the preceding specific embodiments are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

[0093] The entire disclosure of all applications, patents and publications, cited above and below, are hereby incorporated by reference.

[0094] From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

What is claimed is:

1. A method for the modulation of the activity of nitric oxide synthase (NOS), comprising administering at least

(1) folic acid or a folate; and

(2) tetrahydrobiopterin (BH_4)

or derivatives thereof

2. The method as defined in claim 1 wherein folio acid or a folate or a derivative thereof is folio acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or salt thereof or a combination of two or more thereof.

3. The method as defined in claim 2 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methylenetatrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

salts thereof, or a combination of two or more thereof.

4. The method as defined in claim 3 wherein folate or a derivative thereof is in the natural diastereoisomer of folate or a derivative thereof.

5. A method of preventing or treating diseases associated with disturbed activity of nitric oxide synthase (NOS) in the human body comprising administering a therapeutically effective amount of a drug containing at least

(1) folic acid or a folate; and

(2) tetrahydrobiopterin (BH_4)

or derivatives thereof to a human subject.

6. The method as defined in claim 5 wherein folic acid or a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof, or a combination of two or more thereof.

7. The method as defined in claim 6 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

8. The method as defined in claim 7 wherein at least one of folate or tetrahydrobiopterin or a derivative thereof is in the natural stereoisomeric form.

9. A method for the treatment or prevention of at least moderately reduced levels of nitric oxide (NO), comprising administering a therapeutically effective amount of a

drug containing at least

(1) folic acid or a folate; and

(2) tetrahydrobiopterin (BH_4)

or derivatives thereof

10. The method as defined in claim 9 wherein folic acid or a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof ,or a combination of two or more thereof.

11. The method as defined in claim 10 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

12. The method as defined in claim 11 wherein at least one of folate or tetrahydrobiopterin or a derivative thereof is in the natural stereoisomeric form.

13. A method for the treatment or prevention of at least moderately elevated levels of superoxide, comprising administering a therapeutically effective amount of a drug containing at least

(1) folic acid or a folate; and

(2) tetrahydrobiopterin (BH_4)

or derivatives thereof

14. The method as defined in claim 13 wherein folic acid or a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof ,or a combination of two or more thereof.

15. The method as defined in claim 14 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methylenetetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

16. The method as defined in claim 15 wherein at least one of folate or tetrahydrobiopterin or a derivative thereof is in the natural stereoisomeric form.

17. A method of preventing or treating disease associated with decreased nitric oxide levels in the human body comprising administering a therapeutically effective amount of a drug containing at least

(1) folic acid or a folate; and

(2) tetrahydrobiopterin (BH_4)

or derivatives thereof to a human subject.

18. The method as defined in claim 17 wherein folic acid or a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folio acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof ,or a combination of two or more thereof.

19. The method as defined in claim 18 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methylenetetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

20. The method as defined in claim 19 wherein at least one of folate or tetrahydrobiopterin or a derivative thereof is in the natural stereoisomeric form.

21. A method of preventing or treating disease associated with elevated superoxide levels in the human body comprising administering a therapeutically effective amount of a drug containing at least

(1) folic acid or a folate; and

(2) tetrahydrobiopterin (BH_4)

or derivatives thereof to a human subject.

22. The method as defined in claim 21 wherein folic acid or a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof ,or a combination of two or more thereof.

23. The method as defined in claim 22 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methylenetetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

24. The method as defined in claim 23 wherein at least one of folate or tetrahydrobiopterin or a derivative thereof is in the natural stereoisomeric form.

25. A method according to claims 1, 5, 9, 13, 17 or 21 where pathophysiological conditions are present.

26. A method according to claim 25, wherein the disease is a cardiovascular disease.

27. The method as defined in claim 26 wherein folic acid or a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof ,or a combination of two or more thereof.

28. The method as defined in claim 27 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methylenetetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

29. The method as defined in claim 28 wherein at least one of folate or tetrahydrobiopterin or a derivative thereof is in the natural stereoisomeric form.

30. A method according to claim 25 wherein the disease is a neurological disorder.

31. The method as defined in claim 30 wherein folic acid, a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof ,or a combination of two or more thereof.

32. The method as defined in claim 31 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methylenetetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

39. The method as defined in claim 32 wherein at least one of folate or tetrahydrobiopterin or a derivative thereof is In the natural stereoisomeric form.

34. A method according to claims 1, 5, 9, 13, 17, 21, 25, 26 or 30 wherein folic acid or a folate and tetrahydrobiopterin (BH4) or derivatives thereof is administered in combination with at least one active substance or at least one adjuvant substance.

35. A method according to claim 34, wherein the active substance is a pharmaceutically compatible active substance.

36. A method according to claim 35, wherein the pharmaceutically compatible active substance comprises at least arginine.

37. The method as defined in claim 36 wherein folic acid or a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof ,or a combination of two or more thereof.

38. The method as defined in claim 37 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methylenetetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

39. The method as defined in claim 38 wherein at least one of folate or tetrahydrobiopterin or arginine or a derivative thereof is in the natural stereoisomeric form of folate or tetrahydrobiopterin or arginine or a derivative thereof.

40. A method for enhancing the activity of tetrahydrobiopterin (BH_4), comprising administering folic acid, a folate or a derivative thereof.

41. The method as defined in claim 40 wherein folic acid, a folate or a derivative thereof is folic acid (pteroylmonoglutamate), one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salt thereof ,or a combination of two or more thereof.

42. The method as defined in claim 41 wherein folate or a derivative thereof is

dihydrofolic acid,

tetrahydrofolic acid,

5-methyltetrahydrofolic acid,

5,10-methylenetetrahydrofolic acid,

5,10-methenyltetrahydrofolic acid,

5,10-formiminotetrahydrofolic acid,

5-formyltetrahydrofolic acid (leucovorin),

10-formyltetrahydrofolic acid,

10-methyltetrahydrofolic acid,

pharmaceutically compatible salts thereof, or a combination of two or more thereof.

43. The method as defined in claim 42 wherein at least one of folate or tetrahydrobiopterin or a derivative thereof is in the natural stereoisomeric form.

44. A pharmaceutical combination comprising at least

(1) folic acid or a folate; and

(2) tetrahydrobiopterin (BH_4)

or derivatives thereof

45. The pharmaceutical combination as defined in claim 44 wherein folic acid or a folate and tetrahydrobiopterin (BH_4) or derivatives thereof are present in one dosage Within the range from about 0.1 to about 200 mg.

46. A pharmaceutical combination comprising at least

(1) folic acid or a folate; and

(2) tetrahydrobiopterin (BH_4); and

(3) arginine,

or derivatives thereof

47. The pharmaceutical combination as defined in claim 46 wherein folic acid, folate or a derivative thereof and tetrahydrobiopterin (BH_4) and arginine are present in one dosage within the range from about 0.1 to about 200 mg.

48. The pharmaceutical combination as defined in claim 46 wherein at least one of folate or tetrahydrobiopterin or arginine or a derivative thereof is in the natural stereoisomeric form.

* * * * *

EXHIBIT S

Archives of Disease in Childhood, 1978, 53, 674–683

Short reports

Tetrahydrobiopterin therapy of atypical phenylketonuria due to defective dihydrobiopterin biosynthesis

Phenylalanine-4-hydroxylase, tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase need 5, 6, 7, 8-tetrahydro-L-biopterin (BH_4) as a coenzyme. BH_4 is formed *in vivo* from guanosine-triphosphate by several enzymatic steps leading to dihydrobiopterin (DHB). The latter is transformed into (6 S)—or (6 R)—BH_4 by the action of dihydropteridine reductase (DHPR). The lack of any one of the enzymes involved in the BH_4 biosynthesis results in a deficiency of BH_4 leading biochemically to non-functional hydroxylating systems and clinically to variant forms of phenylketonuria (PKU) (Kaufman *et al.*, 1975; Bartholomé *et al.*, 1977). This paper concerns an infant with atypical PKU, who was treated successfully with chemically pure tetrahydrobiopterin.

Case report

A baby girl weighed 2210 g at birth. The Guthrie test for PKU was negative on the 6th day of life, despite correct blood sampling and sufficient protein intake. At age 6 months severe muscle hypotonia and mental retardation were observed. At this time the serum phenylalanine (McCaman and Robins, 1962) was 20 mg/100 ml (1·21 mmol/l). A repeat test of the preserved Guthrie test paper confirmed <2 mg/100 ml (0·121 mmol/l), indicating that a very slow rise in blood phenylalanine had occurred after birth. A low phenylalanine diet was started. After 3 months no clinical improvement was observed and, therefore, further diagnostic procedures were initiated. The phenylalanine-4-hydroxylase activity in a liver biopsy (Bartholomé *et al.*, 1975) was 22 µmol/g protein per hour (normal mean ± SD: 35·2 ± 11·1). K_m for phenylalanine was 0·035 mmol/l (normal). DHPR was 67 nmol NADH/mg protein per min (normal, 70·2–91·1, Bartholomé *et al.*, 1977, courtesy of S. Milstien and S. Kaufman). The phenylalanine-4-hydroxylase *in vivo* test (Curtius *et al.*, 1972, 1977; Zagalak *et al.*, 1977), showed only 2% of the activity found in a control group.

Tetrahydrobiopterin administration, IV and oral

After having received the consent of the parents, the diet was interrupted and a therapeutic trial with BH_4 was begun. For four days on a normal diet (about 120 mg phenylalanine/kg per day) the serum phenylalanine increased from 2·1 to 20·4 mg/100 ml (0·127 to 1·234 mmol/l). Chemically pure (6 R, S)—BH_4· 2 HCl, synthesised by Schircks *et al.* (1977) was administered intravenously (25 mg BH_4· 2 HCl, corresponding to 2·5 mg/kg, in 2 ml isotonic buffer of pH 3·0, containing 25 mg ascorbic acid, 20 mg lactic acid, pH adjusted with 1 N NaOH). Three hours later, the serum phenylalanine decreased to 2·1 mg/100 ml. Six hours and 24 hours after the injection, the phenylalanine values were still 0·9 and 2·1 mg/100 ml (0·05 and 0·127 mmol/l) respectively. 24 hours after the first injection, a second injection of BH_4 was given. The serum phenylalanine remained below 2·1 mg/100 ml during the next 36 hours.

In a second therapeutic trial, 25 mg BH_4· HCl were administered twice within 6 days (Figure) through a gastric tube. Again BH_4 was supplemented with ascorbic acid (1:4, w/w) and dissolved in 20 ml water deaerated with N_2. A striking decrease of the serum phenylalanine concentration was also observed. The therapeutic trial was then discontinued and the phenylalanine-restricted diet was restarted, together with L-dopa, carbidopa, and 5-hydroxytryptophan (Bartholomé *et al.*, 1977).

Discussion

The following findings strongly suggest a defective biosynthesis of dihydropterin in our patient: normal activities of the apoenzymes of phenylalanine-4-hydroxylase and DHPR in liver biopsy; abnormal *in vivo* assay of phenylalanine-4-hydroxylase with a clinical picture similar to PKU; and the patient's prompt biochemical response to the administration of BH_4. Moreover, the synthetic BH_4, given intravenously, proved to have a potent effect in lowering the phenylalanine concentration in blood to normal values in this patient. The fact that it was equally effective when

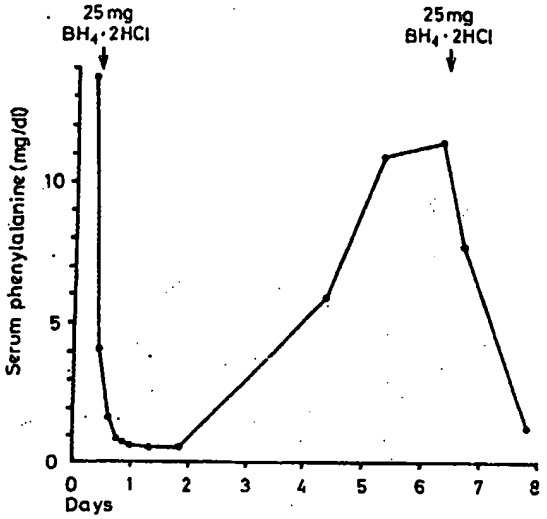

Figure *Decrease of serum phenylalanine concentration after oral administration of $BH_4 \cdot 2$ HCl-ascorbic acid* (1:4, w/w). *The child was on a normal diet. The first administration was before, and the second after a meal of milk.*

given orally proves that BH_4 is absorbed by the intestine.

In a patient with suspected DHB reductase deficiency, Danks *et al.* (1975) found a rather low response to IV administration of a BH_4 preparation, and no response at all to oral administration of 1 mg BH_4.

BH_4 is an essential coenzyme, not only for the formation of tyrosine from phenylalanine in liver, but also for the formation of catecholamines and serotonin which occurs at the synapses within the brain. A patient with a defect in the biosynthesis of BH_4 would lack these biogenic amines in the brain if the BH_4 administered could not penetrate the blood–brain barrier. Using the same BH_4 preparation as Danks *et al.* (1975) in experiments on rats, Kettler *et al.* (1974) thought the evidence suggested that BH_4 did not penetrate the brain. However, the BH_4 preparation used by these authors presumably had low biological activity, and the question of the penetration of BH_4 into the brain must be considered. This being so, patients with defective BH_4 biosynthesis should be treated not only with BH_4 but also with L-dopa, carbidopa, and 5-hydroxytryptophan. We conclude that it should be possible to replace the low phenylalanine diet for such patients, with a normal diet and a BH_4 supplement.

Summary

A patient with atypical phenylketonuria (defective BH_2 synthesis), detected at age 6 months because of severe muscle hypotonia and serum phenylalanine of 20 mg/100 ml, had normal activities of phenylalanine-4-hydroxylase and DHPR in liver biopsy, but only 2% activity in the phenylalanine-4-hyroxylase *in vivo* test using deuterated phenylalanine. After IV administration of 2·5 mg/kg chemically pure tetrahydrobiopterin bis hydrochloride ($BH_4 \cdot 2$ HCl), serum phenylalanine decreased from 20·4 to 2·1 mg/100 ml within 3 hours. Administration of 25 mg $BH_4 \cdot$ HCl and 100 mg ascorbic acid through a gastric tube decreased serum phenylalanine from 13·7 to $\leq$1·6 mg/100 ml within 3 hours and it remained $\leq$2 mg/100 ml for 2 days.

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References

Bartholomé, K., Byrd, D. J., Kaufman, S., and Milstien, S. (1977). Atypical phenylketonuria with normal phenylalanine hydroxylase and dihydropteridine reductase activity *in vitro*. *Pediatrics*, **59**, 757–761.

Bartholomé, K., Lutz, P., and Bickel, H. (1975). Determination of phenylalanine hydroxylase activity in patients with phenylketonuria and hyperphenylalaninemia. *Pediatric Research*, **9**, 899–903.

Curtius, H-Ch., Völmin, J. A., and Baerlocher, K. (1972). The use of deuterated phenylalanine for the elucidation of the phenylalanine-tyrosine metabolism. *Clinica chimica acta*, **37**, 277–285.

Curtius, H-Ch., Zagalak, M-J., Baerlocher, K., Schaub, J., Leimbacher, W., and Redweik, U. (1977). *In vivo* studies of the phenylalanine-4-hydroxylase system in hyperphenylalaninemics and phenylketonurics. *Helvetica paediatrica acta*, **32**, 461–469.

Danks, D. M., Cotton, R. G. H., and Schlesinger, P. (1975). Tetrahydrobiopterin treatment of variant forms of phenylketonuria. *Lancet*, **2**, 1043.

Kaufman, S., Holtzman, N. A., Milstien, S., Butler, I. J., and Krumholz, A. (1975). Phenylketonuria due to a deficiency of dihydropteridine reductase. *New England Journal of Medicine*, **293**, 785–790.

Kettler, R., Bartholini, G., and Pletscher, A. (1974). *In vivo* enhancement of tyrosine hydroxylation in rat striatum by tetrahydrobiopterin. *Nature*, **249**, 476–478.

McCaman, M., and Robins, E. (1962). Fluorimetric method for the determination of phenylalanine in serum. *Journal of Laboratory and Clinical Medicine*, **59**, 885–890.

Schircks, B., Bieri, J. H., and Viscontini, M. (1977). Eine neue, regiospezifische Synthese von L-Biopterin. *Helvetica chimica acta*, **60**, 211.

Zagalak, M-J., Curtius, H-Ch., Leimbacher, W., and Redweik, U. (1977). Quantitation of deuterated and non-deuterated phenylalanine and tyrosine in human plasma using the selective ion monitoring method with combined

gas chromatography-mass spectrometry. Application to the *in vivo* measurement of phenylalanine-4-monooxygenase activity. *Journal of Chromatography*, **142**, 523–531.

J. SCHAUB*, S. DÄUMLING*, H-CH. CURTIUS†, A. NIEDERWIESER†, K. BARTHOLOMÉ§, M. VISCONTINI‡, B. SCHIRCKS‡, AND J. H. BIERI‡

**Children's Hospital, University of Munich, Lindwurmstr. 4, D-8000 Munich, W. Germany*; †*Children's Hospital, University of Zurich, Switzerland*; §*Children's Hospital, University of Heidelberg, W. Germany*; ‡*Chemical Institute, University of Zurich, Switzerland*

Correspondence to Dr J. Schaub.

Diagnosis of phaeochromocytoma after ingestion of imipramine

We describe a case of phaeochromocytoma of the adrenal medulla in one of identical twin girls, diagnosed after the ingestion of imipramine.

Case report

An 11-year-old girl was admitted to hospital with a history of pallor and profuse sweating for 24 hours after a single dose of imipramine syrup (Tofranil 50 mg). There had been a similar episode 2 months earlier after medication with a tablet of imipramine (50 mg). This drug had been prescribed by the family doctor for the treatment of nocturnal enuresis of a year's duration. After the first dose the girl had sweated profusely for about 12 hours. Her mother stopped her medicine but decided to reintroduce it 2 months later as bed-wetting had persisted. Within 6 hours the girl developed the symptoms which led to her admission.

On examination she was 6 cm shorter and 5 kg lighter than her twin, despite being slightly taller and heavier a year earlier. She was pale, cold, and drenched in sweat, with a temperature of 36·2°C. Her pupils were widely dilated but briskly reactive to light, and she had a pulse rate of 160/min. Her blood pressure was 110/85 mmHg and there were no other abnormal findings on physical examination. A random blood sugar was within normal limits and ECG showed sinus tachycardia.

By the next day her diastolic pressure had risen to 100 mmHg and she continued in a state of sympathetic overactivity. A phaeochromocytoma was suspected.

Investigations included measurement of urinary hydroxy-methoxy-mandelic acid (HMMA) and metadrenaline levels. Both levels were raised at 30 μmol/24 h and 45·9 μmol/24 h respectively. Aortography confirmed the presence of a right-sided adrenal tumour. Her hypertension was controlled with oral phenoxybenzamine, and adrenalectomy was performed with full anaesthetic precautions (Crout and Brown, 1969). Histological examination of the tumour confirmed the diagnosis of phaeochromocytoma. After surgery her blood pressure returned to normal and there was no further enuresis. Urinary HMMA and metadrenaline levels fell to normal. The screening test for phaeochromocytoma was normal in her twin sister.

Discussion

Phaeochromocytoma is a rare condition and has been found once in a thousand necropsies in adults (Herman and Mornex, 1964). It is estimated that 0·5% of adult hypertensive patients in the USA have this tumour. For every 10 patients successfully treated, it is thought that there is one death from hypertensive crisis in a patient whose phaeochromocytoma is demonstrated at necropsy (Harrison, 1976). This tumour is considerably rarer in children (Wotherspoon *et al.*, 1974), most reports being of single cases. Symptoms of the disease may be provoked by agents which stimulate release of amines, thereby producing abnormal pressor responses in the tumour. Such pressor agents may be used in provocative tests for the diagnosis of phaeochromocytoma, and include histamine, tetraethylammonium, and methacholine (Beeson and McDermott, 1971).

Imipramine is a tricyclic antidepressant which is commonly used to treat nocturnal enuresis in children. It possesses anticholinergic properties, and reported side effects include dryness of the mouth, dizziness, tachycardia, palpitations, and urinary retention. Excessive sweating has been reported although the mechanism is not known. Tricyclic antidepressants have also been shown to enhance the effects of catecholamines by blockade of active transport from extracellular fluid to cytoplasmic mobile pool (Axelrod *et al.*, 1961).

Our patient may have had an idiosyncratic reaction to imipramine. This may have been the result of direct adrenal stimulation by the drug, or in response to excess catecholamine production after blockade in the re-uptake mechanism by imipramine. We are unaware of any previous report of phaeochromocytoma diagnosed after the ingestion of imipramine.

Oral Administration of Tetrahydrobiopterin Prevents Endothelial Dysfunction and Vascular Oxidative Stress in the Aortas of Insulin-Resistant Rats

Kazuya Shinozaki, Yoshihiko Nishio, Tomio Okamura, Yuichi Yoshida, Hiroshi Maegawa, Hideto Kojima, Masahiro Masada, Noboru Toda, Ryuichi Kikkawa, Atsunori Kashiwagi

Abstract—We have reported that a deficiency of tetrahydrobiopterin (BH_4), an active cofactor of endothelial NO synthase (eNOS), contributes to the endothelial dysfunction through reduced eNOS activity and increased superoxide anion (O_2^-) generation in the insulin-resistant state. To further confirm this hypothesis, we investigated the effects of dietary treatment with BH_4 on endothelium-dependent arterial relaxation and vascular oxidative stress in the aortas of insulin-resistant rats. Oral supplementation of BH_4 (10 mg · kg^{-1} · d^{-1}) for 8 weeks significantly increased the BH_4 content in cardiovascular tissues of rats fed high levels of fructose (fructose-fed rats). Impairment of endothelium-dependent arterial relaxation in the aortic strips of the fructose-fed rats was reversed with BH_4 treatment. The BH_4 treatment was associated with a 2-fold increase in eNOS activity as well as a 70% reduction in endothelial O_2^- production compared with those in fructose-fed rats. The BH_4 treatment also partially improved the insulin sensitivity and blood pressure, as well as the serum triglyceride concentration, in the fructose-fed rats. Moreover, BH_4 treatment of the fructose-fed rats markedly reduced the lipid peroxide content of both aortic and cardiac tissues and inhibited the activation of 2 redox-sensitive transcription factors, nuclear factor-κB and activating protein-1, which were increased in fructose-fed rats. The BH_4 treatment of control rats did not have any significant effects on these parameters. These results indicate that BH_4 augmentation is essential for the restoration of eNOS function and the reduction of vascular oxidative stress in insulin-resistant rats. (*Circ Res.* 2000;87:566-573.)

Key Words: tetrahydrobiopterin ■ insulin ■ endothelium ■ free radicals

It is well known that insulin resistance contributes to the development of atherosclerosis.[1–4] Insulin-resistant states such as hypertension[5] and obesity[6] have been reported to be associated with defective insulin-mediated and endothelium-dependent vasodilation. It has also been shown that insulin-resistant nondiabetics without angiographically detectable coronary atherosclerosis have abnormal endothelial function,[7] suggesting that the endothelium-derived NO system is impaired before the development of overt atherosclerosis. However, the mechanism by which the insulin-resistant state might impair endothelium-dependent coronary vasodilation has not been elucidated.

We recently demonstrated that endothelial dysfunction in the insulin-resistant state is characterized by decreased endothelial production of NO as well as excess production of superoxide anion (O_2^-), resulting in the degradation of NO before it can reach to vascular smooth muscle cells.[8] As previously suggested, (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4) is an important allosteric effector of NO synthase (NOS) through stabilization of the dimeric, an active form of the enzyme, and may play a key role in the control of the calcium-dependent production of NO and O_2^- in vivo.[9] An insufficiency of BH_4 leads to uncoupling of the L-arginine–NO pathway, resulting in increased formation of oxygen radicals by NOS and reduced NO production in vitro.[10–12] Interestingly, we have shown that insulin stimulates the synthesis of BH_4 through the activation of GTP cyclohydrolase I, the rate-limiting enzyme in the de novo synthesis of BH_4 in the aortic endothelium, and that BH_4 synthesis is decreased in the insulin-resistant state.[8] Thus, reduced NO production due to an insufficient amount of BH_4 may be responsible for abnormal vasomotion in the insulin-resistant state. Excess O_2^- reacts with NO and further limits the biologic activity of endothelial NOS (eNOS).[13] Moreover, O_2^- leads to the formation of hydroxyl radicals, which may be cytotoxic to endothelial cells through the direct peroxidation of either lipids or proteins.[14] In the present study, to further confirm the significance of vascular BH_4 content for abnormal endothelial dysfunction in the insulin-resistant state, we investigated the effects of the oral administration of BH_4 on

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From the Third Department of Medicine (K.S., Y.N., H.M., H.K., R.K., A.K.) and Department of Pharmacology (T.O., N.T.), Shiga University of Medical Science, Otsu, Japan; and Laboratory of Biochemistry (Y.Y., M.M.), Faculty of Horticulture, Chiba University, Matsudo, Japan.

Correspondence to Dr Atsunori Kashiwagi, Third Department of Medicine, Shiga University of Medical Science, Tsukinowa-cho, Seta, Otsu, Shiga 520-2192, Japan. E-mail kasiwagi@belle.shiga-med.ac.jp

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TABLE 1. Metabolic Effects of Tetrahydrobiopterin Treatment on Fructose-Induced Insulin Resistance

	Control Group	Control+BH_4 Group	Fructose Group	Fructose+BH_4 Group
Weight, g	338±8.6	340±8.8	344±8.2	342±8.0
Glucose, mg/dL	91.2±1.6	94.8±7.5	96.2±1.2	95.6±6.0
Insulin, μU/mL	16.6±2.9	18.0±4.2	31.2±2.4*§	24.0±1.7
Total cholesterol, mg/dL	52.8±6.2	49.8±3.0	69.8±6.2	66.6±3.6
Triglycerides, mg/dL	65.6±10.6	68.0±7.8	188.4±18.9‡¶	148.4±36.2
Systolic BP, mm Hg	115.4±2.7	113.8±2.5	146.4±2.1‡‖	131.0±2.3**
Diastolic BP, mm Hg	61.6±7.6	63.6±2.9	83.4±2.9†¶	77.2±2.6
SSPG, mg/dL	121±5	128±6	196±10‡‖	162±5**

Control+BH_4 indicates rats fed a standard chow supplemented with 10 mg · kg^{-1} · d^{-1} BH_4; Fructose, rats fed a high-fructose chow; Fructose+BH_4, rats fed a high-fructose chow with 10 mg · kg^{-1} · d^{-1} BH_4; BP, blood pressure.
*$P<0.05$, †$P<0.01$, ‡$P<0.001$ vs Control Group. §$P<0.05$, ¶$P<0.01$, ‖$P<0.001$ vs Control+BH_4 Group.
**$P<0.05$ vs Fructose Group. Values are mean±SEM.

the endothelium-dependent vasorelaxation, endothelial NO and O_2^- production, and oxidative stress–related activation of transcription factors and membrane lipid peroxidation in cardiovascular tissues of insulin-resistant rats.

Materials and Methods

Sapropterin hydrochloride, chemically synthesized BH_4, was a gift of Suntory Ltd. Acetylcholine chloride was purchased from Dai-ichi Pharmaceutical Co. Papaverine hydrochloride was obtained from Dainippon Co. Concanavalin A (ConA)-Sepharose was obtained from Amersham Pharmacia Biotech. L-[^{3}H]Arginine and [^{32}P]dCTP were purchased from New England Nuclear Research Products. Nitrate ion standard solution was obtained from Kanto Chemical Co. All other materials were reagent grade and were purchased from Sigma Chemical Co.

Animals

Male Sprague-Dawley rats (Japan SLC Inc) weighing 150 g were housed in an environmentally controlled room with a 12-hour light/dark cycle and free access to laboratory chow and water. The animals were divided into 4 groups and fed ad libitum 1 of the following diets for 8 weeks: (1) standard chow (control rats), (2) standard chow supplemented with 10 mg · kg^{-1} · d^{-1} sapropterin hydrochloride (BH_4), (3) a diet high in fructose, or (4) a diet high in fructose with 10 mg · kg^{-1} · d^{-1} BH_4. The normal chow (ORIENTAL YEAST) consisted of 58% carbohydrate (no fructose), 12% fat, and 30% protein (N/N). The high-fructose diet (ORIENTAL YEAST) contained 67% carbohydrate (of which 98% was fructose), 13% fat, and 20% protein by energy percent. The animals were administered an intraperitoneal injection of sufficient sodium pentobarbital for anesthesia before they were killed. Both systolic and diastolic blood pressure measurements were made with the tail-cuff method with an electrosphygmomanometer after the rats were prewarmed for 15 minutes.[15] Insulin sensitivity was measured according to the steady-state plasma glucose (SSPG) method with the use of somatostatin, as originally described by Harano et al.[16]

Biopterin Content, GTP Cyclohydrolase I, and Dihydropteridine Reductase Activities

Measurements of biopterin content were performed with HPLC analysis as previously described.[8,17,18] The amount of BH_4 was estimated from the difference between the total (BH_4 plus BH_2 plus oxidized biopterin) and alkaline-stable biopterin (BH_2 plus oxidized biopterin). GTP cyclohydrolase I activity was assayed according to the HPLC method with measurement of neopterin, which was

TABLE 2. Aortic Biopterin Contents and Plasma and Erythrocyte Biopterin Levels in Study Animals

	Control Group	Control+BH_4 Group	Fructose Group	Fructose+BH_4 Group
Aortic content				
BH_4, pmol/mg protein	5.19±0.14	5.93±0.05†	4.71±0.13*¶	6.16±0.10†,††
7,8-BH_2+biopterin, pmol/mg protein	0.91±0.02	1.01±0.01	3.13±0.08‡¶	3.21±0.09‡¶
BH_4/7,8-BH_2+biopterin	5.69±0.01	5.84±0.07*	1.50±0.01‡¶	1.92±0.07¶‡††
Plasma concentration				
BH_4, nmol/L	6.44±0.86	10.4±0.66*	6.64±0.28‖	11.1±0.43*††
7,8-BH_2+biopterin, nmol/L	1.61±0.21	1.88±0.11	2.99±0.11†‖	3.68±0.20†‖
BH_4/7,8-BH_2+biopterin	3.98±0.04	5.49±0.04	2.23±0.02‡¶	3.03±0.05‖**
Erythrocyte concentration				
BH_4, nmol/L	14.1±0.52	17.6±0.49*	10.7±0.47*§	18.5±1.6*††
7,8-BH_2+biopterin, nmol/L	3.40±0.13	5.11±0.11	7.20±0.31†	6.39±0.82
BH_4/7,8-BH_2+biopterin	4.16±0.01	3.46±0.17	1.49±0.02*¶	3.22±0.53††

*$P<0.05$, †$P<0.01$, ‡$P<0.0001$ vs Control Group.
§$P<0.05$, ‖$P<0.01$, ¶$P<0.001$ vs Control+BH_4 Group.
**$P<0.01$, ††$P<0.001$ vs Fructose Group.
Statistical analysis among the 4 groups was made with multiple comparison test with ANOVA and Scheffé's post hoc comparison. Values are mean±SEM (n=4).

released from dihydroneopterin triphosphate after oxidation and phosphatase treatment.[17] Dihydropteridine reductase (DHPR), the recycling enzyme that produces BH_4 from BH_2, was assayed according to the method of Arai et al.[19]

Isometric Tension Studies

Isometric tension studies were performed as previously described.[8] The thoracic aorta (0.6- to 0.8-cm outside diameter) was isolated and cut into strips with special care taken to preserve the endothelium. The strips were partially precontracted with L-phenylephrine. After a plateau was attained, the strips were exposed to acetylcholine, the calcium ionophore A23187, or sodium nitroprusside to construct dose-response curves, which were corrected with the maximal relaxation induced by 100 μmol/L papaverine. In some strips, the endothelium was removed through gentle rubbing of the intimal surface with a cotton ball.

Measurements of NOS Activity and NO Content in Aortic Endothelial Cells

Endothelial NOS activity was measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as previously described.[8,20] The Ca^{2+}-dependent enzyme (eNOS) activity was determined as the difference between the L-[^{3}H]citrulline generated from control samples without EGTA and from those that contained 3 mmol/L EGTA. The concentration of NO in the aortic tissues was determined with a highly sensitive NO measurement system (FES-450; Scholar-Tec Co Ltd) as previously described.[8]

Measurement of Ex Vivo Aortic O_2^- Production

O_2^- production in aortic segments was measured according to the lucigenin-enhanced chemiluminescence method.[8,21,22] Segments of the thoracic aorta (20 mm) were isolated as described earlier, placed in modified Krebs/HEPES buffer (pH 7.4), and allowed to equilibrate for 30 minutes at 37°C. After 5 minutes of dark adaptation, scintillation vials that contain 2 mL Krebs/HEPES buffer with 50 μmol/L lucigenin were placed into a scintillation counter (TRI-CARB1500; Packard Instrument Co) switched to the out-of-coincidence mode. Lucigenin counts were expressed as cpm/mg dry wt vessel. More than 90% and 80% of the chemiluminescence were inhibited with the pretreatment of arterial segments with either 10 μmol/L Tiron (which is a cell-permeable scavenger of O_2^-) and 100 U/mL superoxide dismutase (SOD) (which is a cell-impermeable scavenger of O_2^-), respectively (data not shown). O_2^- production was also measured according to the cytochrome *c* method.[23] The production rate of O_2^- that was inhibited by Cu^{2+},Zn^{2+}-SOD (400 U/mL) was calculated on the basis of the molar extinction coefficient of succinoylated cytochrome *c*.

Measurement of the Lipid Peroxide Content in Cardiovascular Tissues

The lipid peroxide contents of the aortic and cardiac tissues were measured as described previously.[24,25] The lipid fraction of the sample was extracted with the use of a chloroform/methanol solution and resuspended in 100 μL methanol with or without 10 mmol/L triphenylphosphine. After the mixture was incubated for 1 hour at room temperature, 900 μL FOXII reagent[24] was added. The difference of absorbance at 560 nm between the sample with and that without triphenylphosphine was considered to reflect the lipid peroxide content. A standard curve was constructed with hydrogen peroxide.

Electrophoretic Gel Shift Assay

Nuclear extracts were prepared according to our previously described method[25] and stored at −80°C. The DNA probes for nuclear factor-κB (NF-κB), activating protein-1 (AP-1), and specificity protein-1 (Sp-1) (Promega) were labeled with [^{32}P]ATP and T4 polynucleotide kinase. For competition studies, the experimental conditions were identical, except that the appropriate competitor oligonucleotides were added at a 50- to 100-fold molar excess to the reaction mixture before the addition of nuclear extract.

Statistical Analysis

All values are expressed as mean±SEM. The dose-dependent vascular relaxation was compared among the 4 groups with repeated measures ANOVA. Vascular responses were compared among the 4 groups with 2-way ANOVA. Comparisons among those groups were performed with ANOVA with a post hoc Scheffé's comparison. A value of $P<0.05$ was considered statistically significant.

Results

Metabolic Characteristics and Blood Pressure of the Rats

As shown in Table 1, animals fed high levels of fructose showed significant elevations of plasma insulin, triglyceride, blood pressure, and SSPG compared with control rats. On the other hand, the treatment with BH_4 significantly lowered systolic blood pressure and the SSPG level and tended to decrease diastolic blood pressure, insulin, and triglyceride levels in fructose-fed rats. However, this agent did not affect any of these parameters in control rats.

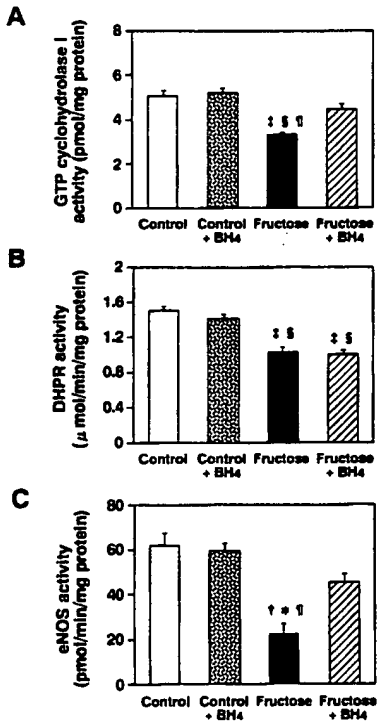

Figure 1. Effects of dietary BH_4 on GTP cyclohydrolase I (A), DHPR (B), and eNOS activity (C) in aortas from control and high fructose–fed rats. Aortic vessels were harvested from rats fed standard chow (Control), standard chow supplemented with 10 mg · kg^{-1} · d^{-1} sapropterin hydrochloride (BH_4) (Control+BH_4), high-fructose chow (Fructose), or high-fructose chow with 10 mg · kg^{-1} · d^{-1} BH_4 (Fructose+BH_4). After 8 weeks, segments of thoracic aorta were harvested and assayed for each enzyme activity as described in Materials and Methods. The activities of GTP cyclohydrolase I, DHPR, and eNOS were expressed as pmol/mg protein, μmol · min^{-1} · mg protein^{-1}, and pmol · min^{-1} · mg protein^{-1}, respectively. Data are expressed as mean±SEM of duplicated determinations from 4 different experiments. †$P<0.01$, ‡$P<0.001$ vs control rats. *$P<0.01$, §$P<0.001$ vs control+BH_4 rats. ¶$P<0.05$ vs fructose+BH_4 rats.

TABLE 3. Effect of Tetrahydrobiopterin Feeding on Nitrogen Oxide (Nitrate Plus Nitrite) Production From Aortic Vessels With Endothelium

	Vascular Nitrogen Oxide (Nitrate Plus Nitrite) Production, pmol · h^{-1} · mg dry wt of $vessel^{-1}$			
Incubation Condition	Control Group	Control+BH_4 Group	Fructose Group	Fructose+BH_4 Group
Buffer alone	48.2±3.5	45.6±2.4	30.5±3.0‖¶	38.6±1.8
+A23187 (10 μmol/L)	134±18.9*	123±12.6†	55.8±5.7†‖¶**	103.8±6.0†
+L-NAME (10 μmol/L) +A23187 (10 μmol/L)	29.5±4.9‡	24.7±2.2§	39.6±2.5	26.5±4.2§

Production of nitrogen oxide (nitrate plus nitrite) from aortic vessels with and without stimulation of A23187. Basal concentrations were measured in the absence of calcium ionophore A23187 (10 μmol/L). Vascular nitrogen oxide release was measured after stimulation with A23187, as well as in the presence of 10 μmol/L L-NAME.

*$P<0.05$, †$P<0.01$ vs the corresponding basal values (buffer alone).

‡$P<0.05$, §$P<0.01$ vs the corresponding values of stimulation with A23187.

‖$P<0.01$ vs the corresponding values of Control Group.

¶$P<0.01$ vs the corresponding values of Control+BH_4 Group.

**$P<0.05$ vs corresponding values of Fructose+BH_4 Group. Values are expressed as mean±SEM (n=5).

Biopterin Content, GTP Cyclohydrolase I, and DHPR Activities

The BH_4 content of both the aorta and erythrocytes in fructose-fed rats were significantly lower than those in control rats (Table 2). In contrast, fructose-fed rats showed 3.4-, 1.9-, and 2.1-fold elevations of 7,8-BH_2 plus biopterin in the aorta, plasma, and erythrocytes compared with control rats, respectively. Rats fed a diet that contained BH_4 demonstrated a significant elevation of BH_4 level compared with the control rats, whereas BH_4 treatment did not significantly alter the content of 7,8-BH_2 plus biopterin.

As shown in Figure 1A, GTP cyclohydrolase I activity in the aortas of fructose-fed rats was significantly lower than that of control rats. Fructose-fed rats treated with BH_4 showed a significant elevation of the enzyme activity compared with fructose-fed rats, whereas the activity in control rats was not affected by the BH_4 treatment. The activity of DHPR, the recycling enzyme that converts BH_2 to BH_4, in the aorta of fructose-fed rats was also significantly lower than that of control rats (Figure 1B). However, the activity in fructose-fed rats was not affected by the BH_4 treatment.

Effects of BH_4 Treatment on NOS Activity and NO Production by Endothelial Cells

The eNOS activity was significantly depressed in fructose-fed rats (from 63.4 to 22.5 pmol · min^{-1} · mg $protein^{-1}$) (Figure 1C). The administration of BH_4 to fructose-fed rats significantly elevated the enzyme activity to 50.1 pmol · min^{-1} · mg $protein^{-1}$, whereas the activity in control rats was not affected by the treatment with BH_4. There was no significant difference of Ca^{2+}-independent NOS activity in the homogenates of aortic endothelial cells among the 4 groups of rats.

As shown in Table 3, after stimulation with A23187, the NO production in fructose-fed rats was significantly increased by the BH_4 treatment. After the preincubation of the vessels with N^G-nitro-L-arginine methyl ester (L-NAME), the A23187-stimulated NO production was reduced approximately to the basal level, and the differences among the 4 groups disappeared.

Superoxide Anion Generation From Aortas With or Without Endothelium

As shown in Table 4, the basal O_2^- production by the aortic segments with endothelium (group B) from fructose-fed rats

TABLE 4. Effect of Tetrahydrobiopterin Feeding on Superoxide Anion Production by Endothelial Nitric Oxide Synthase

			Vascular Superoxide Production, cpm×10^3/mg dry wt of vessel			
Group	Endothelium	Incubation Condition	Control Group	Control+BH_4 Group	Fructose Group	Fructose+BH_4 Group
A	−	Buffer alone	18.4±3.2*	22.8±3.7*	20.4±2.8†	23.5±3.2*
B	+	Buffer alone	27.6±5.7	41.1±12.5	73.4±20.3§	34.5±6.2
C	+	+A23187 (10 μmol/L)	58.3±19.6†	64.9±12.6*	232.6±60.2‖¶**†	67.8±8.2†
D	+	+SOD (100 U/mL)+A23187 (10 μmol/L)	28.2±11.1‡	42.8±6.7‡	47.0±17.7†‡	29.5±5.7‡
E	+	+L-NAME (10 μmol/L)+A23187 (10 μmol/L)	46.4±10.3	54.1±9.7	64.3±12.4‡	55.8±9.2

Basal production was measured without calcium ionophore A23187 (10 μmol/L) in the absence (group A) or presence (group B) of endothelium. Vascular superoxide production was measured after stimulation with A23187 (group C), as well as in the presence of either 100 U/mL Cu^{2+},Zn^{2+}-SOD (group D) or L-NAME (10 μmol/L).

*$P<0.05$, †$P<0.01$ vs the corresponding vessels of group B, with unpaired Student's t test.

‡$P<0.05$ vs the corresponding vessels of group B.

§$P<0.05$ vs Control Group B.

‖$P<0.01$ vs Control Group C.

¶$P<0.01$ vs Control+BH_4 Group C.

**$P<0.01$ vs Fructose+BH_4 Group C.

Statistical analysis among the 4 groups was made with multiple comparison test with ANOVA and Scheffé's post hoc comparison. Values are mean±SEM (n=5).

was significantly higher than that of control rat aorta ($P<0.05$). Removal of the endothelium slightly (33%) reduced the O_2^- level in control vessels, whereas a marked reduction (72%) in O_2^- production was found in the endothelium-denuded vessels of fructose-fed rats (group A). The O_2^- production was significantly increased by A23187 in all groups, and the increase was greater in the fructose-fed rats than in the control rats (group C). BH_4 treatment did not affect basal or A23187-stimulated O_2^- production in control rats. However, in the fructose-fed rats, the A23187-stimulated O_2^- production was significantly decreased to the levels of control rats by the treatment with BH_4. The increase in O_2^- production in fructose-fed rats was abolished, resulting in basal-level production after incubation with either Cu^{2+},Zn^{2+}-SOD (group D) or L-NAME (group E).

To confirm the validity of the lucigenin method for the measurement of O_2^- in our systems, we also measured vascular basal O_2^- production according to the cytochrome *c* method. O_2^- production by aortic segments (n=4) from the fructose-fed rats (3.58±0.33 nmol · min^{-1} · mg^{-1} dry wt vessel) was significantly higher than that of segments from control rats without (1.48±0.28, $P<0.001$) or with (1.10±0.23, $P<0.0001$) BH_4 treatment and than that of segments from BH_4-treated fructose-fed rats (1.85±0.19, $P<0.01$).

Effects of BH_4 Treatment on Vascular Reactivity

The addition of either acetylcholine or A23187 produced a dose-dependent relaxation in aortic strips (Figures 2A and 2B). The maximal response was significantly reduced and the ED_{50} value was increased in the aortas derived from the fructose-fed rats compared with those from the control rats. The dose-relaxation curve in the aortas from BH_4-treated control rats was similar to that of control rats, whereas the curve in the aorta from fructose-fed rats was significantly improved by the BH_4 supplementation. Vasodilator responses to sodium nitroprusside were almost identical among the 4 different groups (Figure 2C). The acetylcholine-induced relaxation in aortic strips from all 4 groups was abolished by either treatment with 10^{-4} mol/L L-NAME or endothelial denudation (data not shown).

Lipid Hydroperoxide Content and Activation of NF-κB and AP-1 in the Aortas and Hearts

The lipid hydroperoxide contents of the aorta and cardiac ventricle from fructose-fed rats were significantly higher than those of the control rats, respectively (Figure 3). The treatment with BH_4 completely restored the content to the control level in the fructose-fed rats. As shown in Figure 4A, the binding of the nuclear extract of the aorta of the fructose-fed rats to an oligonucleotide that contained the NF-κB consensus sequence was markedly increased compared with the binding in the extract from the control rats. However, the treatment of fructose-fed rats with BH_4 restored the level of binding to the control level. The level of binding of the oligonucleotide that contained the NF-κB sequence by the nuclear protein obtained from the hearts of fructose-fed rats was also increased compared with that of the nuclear protein obtained from control rats (Figure 4D). This increase in

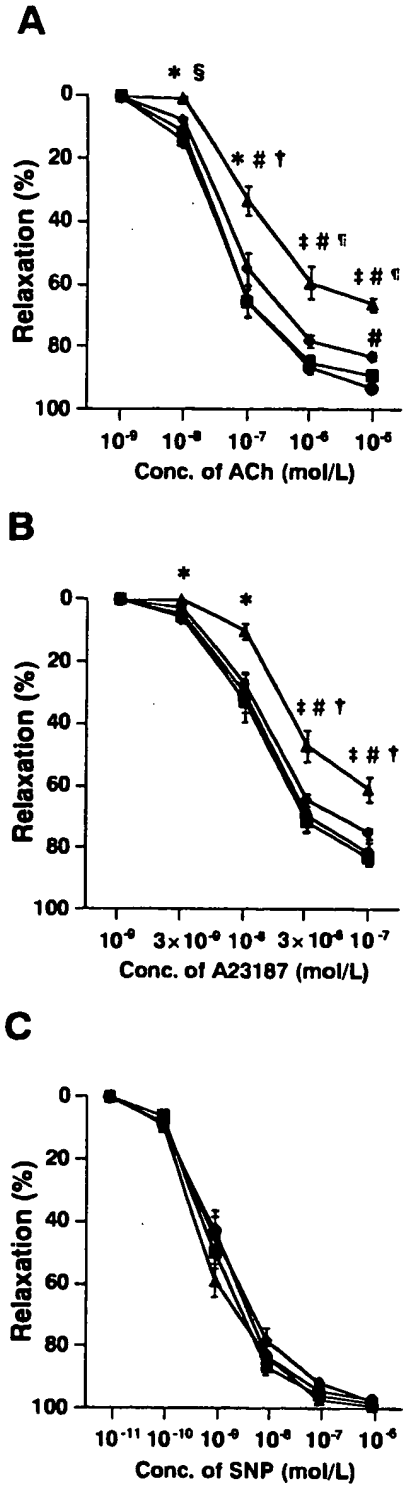

Figure 2. Effects of dietary BH_4 on vasodilator responses to acetylcholine (A), calcium ionophore A23187 (B), and sodium nitroprusside (C) in aortic strips with endothelium from control and high fructose–fed rats. Aortic vessels were harvested from Control (■), Control+BH_4 (●), Fructose (▲), and Fructose+BH_4 (♦) groups for a period of 8 weeks. The strips were partially precontracted with L-phenylephrine. Relaxation induced by 100 μmol/L papaverine was taken as 100%. Data are expressed as mean±SEM (n=6). *$P<0.05$, ‡$P<0.001$ vs Control strips. §$P<0.05$, #$P<0.01$ vs Control+BH_4 strips. †$P<0.05$, ¶$P<0.01$ vs Fructose+BH_4 strips. Data are expressed as mean±SEM (n=6). Conc. indicates concentration of each drug.

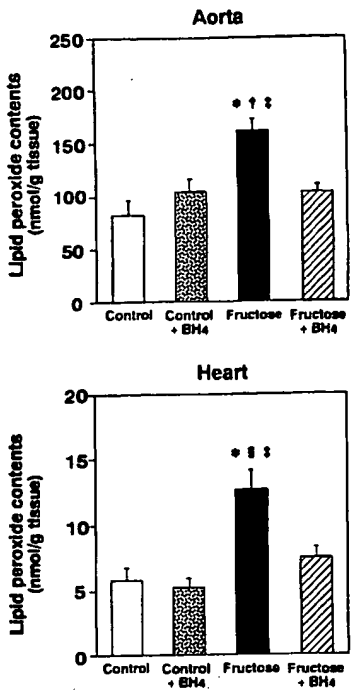

Figure 3. Effects of dietary BH_4 on either aortic (top) or cardiac (bottom) lipid peroxide contents in control and high fructose–fed rats. After 8 weeks, segments of thoracic aorta and heart were harvested and assayed for lipid peroxide content as described in Materials and Methods. Data are expressed as mean±SEM of 4 different experiments. *$P<0.01$ vs Control rats. †$P<0.05$, §$P<0.01$ vs Control+BH_4 rats. ‡$P<0.05$ vs Fructose+BH_4 rats.

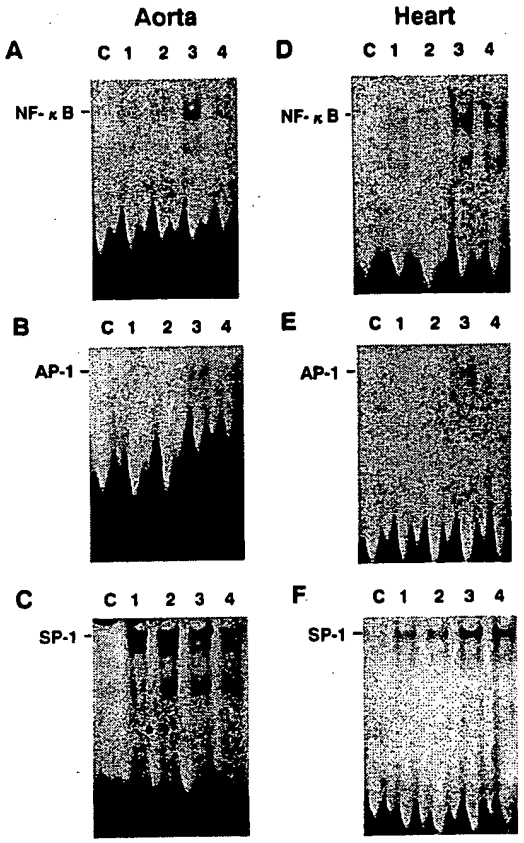

Figure 4. Effects of dietary BH_4 on the transcription activities of NF-κB, AP-1, and Sp-1 in the aortas and hearts of 4 different rats. Electrophoretic mobility shift assay of the transcription factors, which bound to the consensus nucleotide sequence for NF-κB (A and D), AP-1 (B and E), and Sp-1 (C and F), was performed with 2 to 5 μg nuclear protein isolated from either aortas (A through C) or hearts (D through F) of rats, respectively. Lane C shows the addition of excess amount of cold oligonucleotides. Aortic vessels were harvested from Control (lane 1), Control+BH_4 (lane 2), Fructose (lane 3), or Fructose+BH_4 (lane 4). After 8 weeks, segments of thoracic aorta and heart were harvested and prepared for nuclear extracts as described in Materials and Methods.

binding activity was also abolished by the BH_4 treatment (Figure 4D). Consistent with the results for NF-κB, the AP-1 binding of the nuclear extracts from both the aorta and heart from fructose-fed rats was also increased, and treatment with BH_4 also prevented those increases in AP-1 binding (Figures 4B and 4E). In contrast, Sp-1 binding of the nuclear protein from the aorta and heart did not differ among the 4 groups (Figures 4C and 4F). BH_4 treatment did not affect the binding activities of NF-κB or AP-1 in control rats.

Discussion

The oral administration of BH_4 to insulin-resistant rats restored endothelium-dependent vasodilation via the activation of eNOS. Furthermore, the treatment of fructose-fed rats with BH_4 caused a reduction in both endothelial O_2^- generation and lipid peroxide content in cardiovascular tissues. However, the sensitivity of aortic smooth muscle to sodium nitroprusside did not differ among the 4 groups of rats. These results suggest that BH_4 specifically affects endothelium-dependent pathways in insulin-resistant rat vessels. Furthermore, the results demonstrated that the increased binding activity of 2 redox-sensitive transcription factors, NF-κB and AP-1, in insulin-resistant rats was also prevented by the treatment with BH_4.

Biopterin metabolism is critical for the regulation of NOS activity. It has been suggested that depletion of BH_4 and reduction in the BH_4/7,8-BH_2 ratio are critical for the regulation of endothelial production of O_2^- as well as NO.[8,12] In the present study, BH_4 supplementation significantly increased the vascular content of BH_4, restored NO production, and reduced A23187-stimulated O_2^- production in the aortas from fructose-fed rats. Previously, we found that the insulin-resistant state induced a decrement of eNOS activity without affecting the eNOS mRNA expression in the aorta of rats.[8] Consistent with these results, we could not find any increase in either eNOS protein or mRNA expression in the aortas of fructose-fed rats in response to BH_4 supplementation (data not shown). Therefore, it is clear that impaired BH_4 synthesis in the aortas of rats in the insulin-resistant state is closely associated with a decrement in eNOS activity rather than with the expression level of the protein.

An important question that remains to be answered is how the insulin-resistant state affects biopterin metabolism. In mammalian cells, BH_4 is synthesized through 2 distinct pathways: 1 is a de novo synthetic pathway that uses GTP as a precursor, and the other is the regeneration of BH_4 from BH_2 through a pterin salvage pathway.[12] A quinonoid form of BH_2 (qBH_2) is generated when BH_4 is used for NO synthesis.[19] The reduction of qBH_2 to BH_4 proceeds through the action of

DHPR. There are several lines of evidence that suggest the vascular effects of insulin are impaired in various insulin-resistant states, including hypertension, obesity, and diabetes.[5,6,26,27] In the present study, we found that DHPR as well as GTP cyclohydrolase I activities in the endothelial cell were reduced in the insulin-resistant state. Previously, we found that endothelial BH_4 content and the activity of GTP cyclohydrolase I were markedly increased, whereas the levels of BH_2 were markedly decreased, in the aortas of exogenous hyperinsulinemic rats without insulin resistance.[8] Therefore, it appears that insulin stimulates BH_4 synthesis via the activation of GTP cyclohydrolase I and DHPR[28] and that those effects of insulin effect are impaired in the insulin-resistant state. On the other hand, the biosynthesis of BH_4 depends on a normal cellular redox state, and oxidative stress impairs the endothelial recycling of BH_4.[29] The present findings of increased vascular O_2^- production and 7,8-BH_2 levels imply that the increased production of reactive oxygen species in the insulin-resistant state resulted in enhanced oxidation of BH_4. It is therefore possible that both insulin resistance and increased oxidative stress contribute to impaired production of BH_4.

It is still unclear whether the eNOS dysfunction is due to the decreased BH_4 levels or the decreased BH_4/7,8-BH_2 ratio.[12] Under control conditions, BH_4 supplementation did not affect endothelial NO/O_2^- generation or the vasoreactivity to A23187, indicating that the content of intracellularly stored BH_4 is sufficient to maximally activate eNOS. The increase in the ratio of BH_4/7,8-BH_2 in the BH_4-treated fructose-fed rats (Table 2) was smaller than the change in BH_4 content itself. These results suggest that the content of intracellularly stored BH_4 rather than the ratio of BH_4/7,8-BH_2 was a determining factor for the formation of endothelium-derived NO under the conditions of this study.

Insulin resistance causes oxidative stress to cardiovascular tissues and the release of oxygen free radicals from endothelial cells. Consistent with the increased lipid peroxidation of the membrane fraction in fructose-fed rats, both NF-κB and AP-1 were markedly activated in cardiovascular tissues. A previous report also indicated that both NF-κB and AP-1 are activated by oxidative stress.[30] In the present study, treatment with BH_4 normalized the vascular O_2^- production, membrane lipid peroxidation, and NF-κB and AP-1 activation in cardiovascular tissues of insulin-resistant rats. Based on the fact that activation of these transcription factors is related to the alteration of the expression of various atherogenic genes,[27] the present findings suggest that sufficient supplementation with BH_4 might help to prevent or delay the occurrence of cardiovascular diseases in the insulin-resistant state.

Whether the partial improvements of insulin sensitivity and blood pressure in BH_4-treated insulin-resistant rats are primarily associated with restored endothelial function remains unknown, although it is clear that impairment of endothelial function precedes the development of hypertension in the insulin-resistant status.[31] Baron and coworkers have shown that insulin-mediated vasodilation is impaired in patients with insulin resistance[5,6,27] and that the defective insulin-mediated vasodilation accounts for 20% to 30% of the decrement in insulin action (insulin resistance).[32] Therefore, the restoration of endothelial function by BH_4 may contribute to a mechanism to prevent the rise in blood pressure and insulin resistance seen in fructose-fed rats. However, we cannot exclude the possibility that partial restoration of blood pressure by BH_4 supplementation may further improve vascular dysfunction in the insulin-resistant state.

In conclusion, the novel observation in the present study was that the oral administration of BH_4 to insulin-resistant rats restored endothelium-dependent vasodilation and relieved vascular oxidative stress, at least in part through eNOS activation. The impaired endothelial function and the increased oxidative stress in the aorta are due to insufficient synthesis of BH_4, resulting in reduced activity of eNOS. Recent reports have demonstrated that the short-term administration of BH_4 restores endothelial function in hypercholesterolemic humans[33] and smokers.[34] Further studies are required to clarify the usefulness of BH_4 treatment for the prevention of endothelial dysfunction and the development of cardiovascular diseases in insulin-resistant patients.

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References

1. Zavaroni I, Bonora E, Pagliara M, Dall'Aglio E, Luchetti L, Buonanno G, Bonati PA, Bergonzani A, Gundi L, Passeri M, Reaven GM. Risk factors for coronary artery disease in healthy persons with hyperinsulinemia and normal glucose tolerance. *N Engl J Med.* 1989;320:702–706.
2. Reaven GM. Role of insulin resistance in human disease. *Diabetes.* 1988;37:1595–1607.
3. Laakso M, Sarlund H, Salonen R, Suhonen M, Pyörälä K, Salonen JT, Karhapää P. Asymptomatic atherosclerosis and insulin resistance. *Arterioscler Thromb.* 1991;11:1068–1076.
4. Shinozaki K, Naritomi H, Shimizu T, Suzuki M, Ikebuchi M, Sawada T, Harano Y. Role of insulin resistance associated with compensatory hyperinsulinemia in ischemic stroke. *Stroke.* 1996;27:37–43.
5. Baron AD, Brechtel-Hook G, Johnson A, Hardin D. Skeletal muscle blood flow: a possible link between insulin resistance and blood pressure. *Hypertension.* 1993;21:129–135.
6. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD. Obesity/insulin resistance is associated with endothelial dysfunction: implication for the syndrome for insulin resistance. *J Clin Invest.* 1996; 97:2601–2610.
7. Shinozaki K, Szuki M, Ikebuchi M, Takaki H, Hara Y, Tsushima M, Harano Y. Insulin resistance associated with compensatory hyperinsulinemia as an independent risk factor for vasospastic angina. *Circulation.* 1995;92:1749–1757.
8. Shinozaki K, Kashiwagi A, Nishio Y, Okamura T, Yoshida Y, Masada M, Toda N, Kikkawa R. Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through nitric oxide/O_2^- imbalance in insulin-resistant rat aorta. *Diabetes.* 1999;48:2437–2445.
9. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest.* 1997;100:2153–2157.
10. Kwon NS, Nathan CF, Stuehr DJ. Reduced biopterin as cofactor in the generation of nitrogen oxides by murine macrophages. *J Biol Chem.* 1989;264:20446–20501.
11. Reif A, Fröhlich LG, Kotsonis P, Frey A, Bömmel HM, Wink DA, Pfleiderer W, Schmidt HHHW. Tetrahydrobiopterin inhibits monomerization and is consumed during catalysis in neuronal NO synthase. *J Biol Chem.* 1999;274:24921–24929.
12. Wermer ER, Werner-Felmayer G, Wachter H, Mayer B. Biosynthesis of nitric oxide: dependence on pteridine metabolism. *Rev Physiol Biochem Pharmacol.* 1995;127:97–135.
13. Kontos HA, Kontos MC. Role of products of univalent reduction of oxygen in hypertensive vascular injury. In: Laragh JH, Brenner BM, eds. *Hypertension: Pathophysiology, Diagnosis, and Management.* New York, NY: Raven Press; 1995:685–696.

14. Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. *Lab Invest.* 1982;47:412–425.
15. Bunag RD. Validation in awake rats of a tail-cuff method for measuring systolic pressure. *J Appl Physiol.* 1973;34:279–282.
16. Harano Y, Ohgaku S, Kosugi K, Yasuda H, Nakano T, Kobayashi M, Hidaka H, Izumi K, Kashiwagi A, Shigeta Y. Clinical significance of altered insulin sensitivity in diabetes mellitus assessed by glucose, insulin, and somatostatin infusion. *J Clin Endocrinol Metab.* 1981;52: 982–987.
17. Masada M, Akino M, Sueoka T, Katoh S. Dyspropterin, an intermediate formed from dihydroneopterin triphosphate in the biosynthetic pathway of tetrahydrobiopterin. *Biochim Biophys Acta.* 1985;840:235–244.
18. Kaufman S. Metabolism of the phenylalanine hydroxylation cofactor. *J Biol Chem.* 1967;242:3934–3943.
19. Arai N, Narisawa K, Hayakawa H, Tada K. Hyperphenylalanemia due to dihydropteridine reductase deficiency: diagnosis by enzyme assays on dried blood spots. *Pediatrics.* 1982;70:426–430.
20. Rees D, Ben-Ishay D, Moncada S. Nitric oxide and the regulation of blood pressure in the hypertension-prone and hypertension-resistant Sabra rat. *Hypertension.* 1996;28:367–371.
21. Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vascular tone. *J Clin Invest.* 1996;97:1916–1923.
22. Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA. Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem.* 1998;73:2015–2023.
23. Kuthan H, Ullrich V. A quantitative test for superoxide radicals produced in biological systems. *Biochem J.* 1982;203:551–558.
24. Nourooz-Zadeh J, Tjadddini-Sarmadi-J, Wolff-SP. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Anal Biochem.* 1994;220:403–409.
25. Nishio Y, Kashiwagi A, Taki H, Shinozaki K, Maeno Y, Kojima H, Maegawa H, Haneda M, Hidaka H, Yasuda H, Horiike K, Kikkawa R. Altered activity of transcription factors and their related gene expression in cardiac tissues of diabetic rats. *Diabetes.* 1998;47:1318–1325.
26. Kuboki K, Jiang ZY, Takahara N, Ha SW, Igarashi M, Yamauchi T, Feener EP, Herbert TP, Rhodes CJ, King GL. Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo: a specific vascular action of insulin. *Circulation.* 2000;101: 676–681.
27. Laakso M, Edelman SV, Brechtel G, Baron AD. Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man: a novel mechanism for insulin resistance. *J Clin Invest.* 1990;5:1844–1848.
28. Viveros OH, Lee C-L, Abou-Donia MM, Nixon JC, Nichol CA. Biopterin cofactor biosynthesis: independent regulation of GTP cyclohydrolase in adrenal medulla and cortex. *Science.* 1981;213:349–350.
29. Komori Y, Hyun J, Chiang K, Fukuto JM. The role of thiols in the apparent activation of rat brain nitric oxide synthase. *J Biochem.* 1995; 117:923–927.
30. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. *FASEB J.* 1996;10:709–720.
31. Katakam PVG, Ujhelyi MR, Hoenig ME, Miller AW. Endothelial dysfunction precedes hypertension in diet-induced insulin resistance. *Am J Physiol.* 1999;275:R788–R792.
32. Baron AD. Hemodynamic action of insulin. *Am J Physiol.* 1994;267: E187–E202.
33. Stroes E, Kastelin J, Cosentino F, Erkelens W, Wever R, Koomans H, Lüscher TF, Rabelink T. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest.* 1997;99:41–46.
34. Ueda S, Matsuoka H, Miyazaki H, Usui M, Okuda S, Imaizumi T. Tetrahydrobiopterin restores endothelial function in long-term smokers. *J Am Coll Cardiol.* 2000;35:71–75.

EXHIBIT U

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RESEARCH LETTER

Robert Steinfeld · Alfried Kohlschütter
Johannes Zschocke · Martin Lindner · Kurt Ullrich
Zoltan Lukacs

Tetrahydrobiopterin monotherapy for phenylketonuria patients with common mild mutations

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The effect of tetrahydrobiopterin (BH_4) administration was studied in three infants with BH_4 responsive phenylalanine hydroxylase (PAH) deficiency by correlating different oral BH_4 doses with plasma phenylalanine levels under defined protein intake.

Primary hyperphenylalaninaemias are either caused by loss of activity of PAH (EC 1.14.16.1) or by lack of its cofactor (*6R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4). PAH deficiency and disorders of BH_4 metabolism can be differentiated by a BH_4 loading test. Recently, however, several patients with BH_4 responsive PAH deficiency have been described [1, 4, 5, 6].

BH_4 responsiveness was initially demonstrated for our patients 1, 2 and 3, detected on neonatal screening, by oral BH_4 loading tests (data not shown). Cofactor deficiency was excluded by normal urinary pterin concentrations, normal activity of dihydropteridine reductase in erythrocytes and (for patient 2) by normal neurotransmitter concentrations in CSF (data not shown). PAH deficiency was confirmed by finding common PAH gene mutations in all patients: A104D + K320N in patient 1 and Y414C + Y414C in patient 2 suggested a mild PKU phenotype, whereas A403V + A395P in patient 3 suggested a mild hyperphenylalaninaemia phenotype not requiring dietary treatment [2].

Patients 1 and 2 were selected for the investigation of their BH_4 response in detail, to find the optimal BH_4 dose and to explore a possible long-term BH_4 treatment. For this purpose, plasma phenylalanine levels were correlated with different oral BH_4 doses under a protein intake corresponding to 100–150 mg phenylalanine/kg body weight and day (Fig. 1). In patient 1 (Fig. 1A), plasma phenylalanine levels fell remarkably within 12 h after application of 10 mg/kg BH_4. In the case of patient 2, the BH_4 response was slightly different (Fig. 1B) as a daily BH_4 dose of 5 mg/kg body weight was not sufficient to maintain phenylalanine values below 10 mg/dl. Both children with mild PKU (patients 1 and 2) were continued on oral BH_4 supplementation and were fed without protein restriction or special phenylalanine-free formulae. During the past 12 months, plasma phenylalanine concentrations of patients 1 and 2 remained within the desirable range at daily BH_4 doses between 5–10 and 10–20 mg/kg body weight respectively. Both infants have developed normally so far.

We have identified three children with PAH genotypes for which BH_4 responsive hyperphenylalaninaemia has not been previously reported. In addition, we report the first patient (patient 2) with BH_4 sensitivity who is homozygous for one mutation (Y414C). This indicates that PAH heterotetramers as well as PAH homotetramers are compatible with BH_4 responsiveness. Our findings question the concept of decreased cofactor affinity as a valid explanation for BH_4 responsiveness as no defined structural motif responsible for the perturbation of BH_4 affinity can be deduced from published mutations. Surprisingly, different individuals with an identical PAH genotype (R408W/Y414C) have shown divergent BH_4 responsiveness [5]. In fact, two large studies on genotype-phenotype correlation revealed several PAH alleles with inconsistent phenotypes [2,3]. Similarly, homozygosity for the Y414C mutation, the second most common PAH allele in Northern Europe, has not been linked to a BH_4 responsive phenotype before.

Several explanations for these phenomena are possible. Assuming that no enzyme other than PAH is involved in the hydroxylation of phenylalanine to tyrosine, one can consider two effects conferred by a high BH_4

R. Steinfeld · A. Kohlschütter · K. Ullrich · Z. Lukacs (✉)
Department of Pediatrics,
University of Hamburg,
Martinistrasse 52, 20246 Hamburg, Germany
E-mail: lukacs@uke.uni-hamburg.de
Tel.: +49-40-428033735
Fax: +49-40-42806717

J. Zschocke
Institute of Human Genetics,
University of Heidelberg, Heidelberg, Germany

J. Zschocke · M. Lindner
Department of Pediatrics,
University of Heidelberg, Heidelberg, Germany

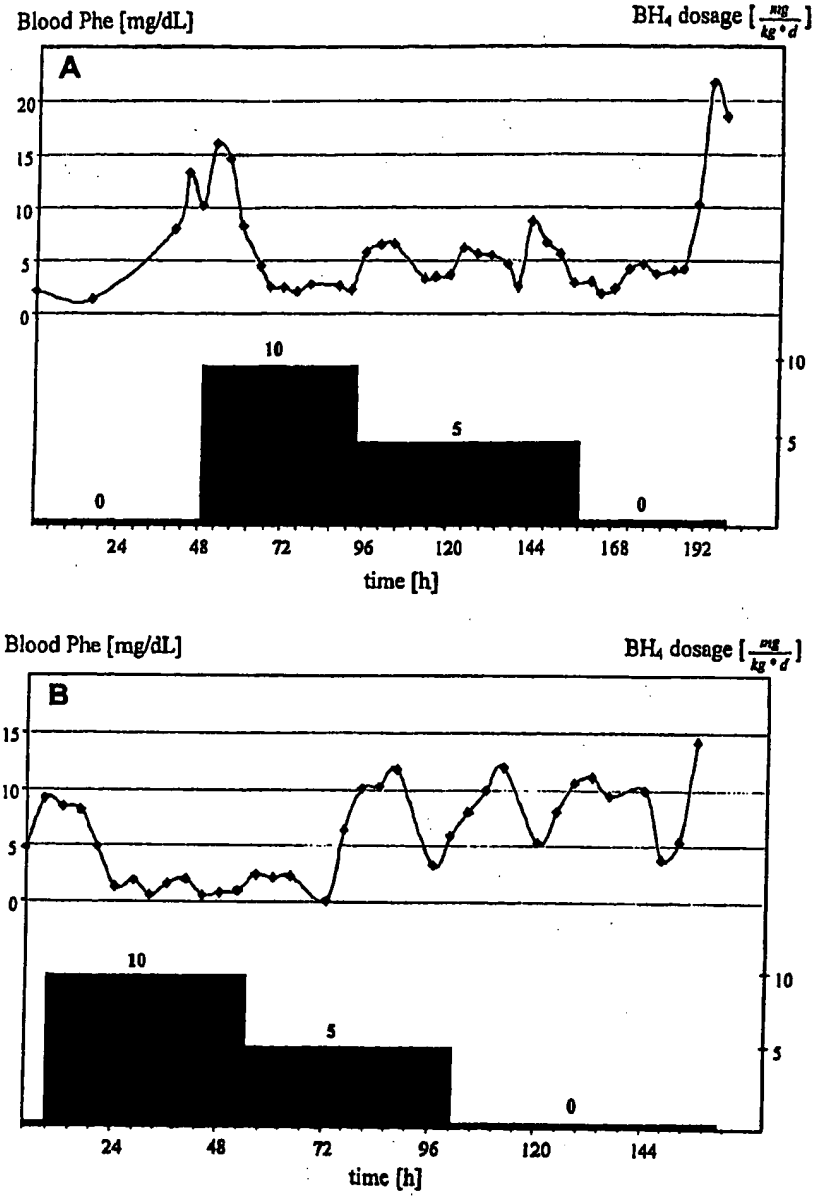

Fig. 1. Response of blood phenylalanine levels to oral BH_4 doses for patients 1 and 2. The daily protein intake was 100–150 mg/kg body weight for both patients. Samples were taken and analysed for phenylalanine content (*filled diamonds*) approximately every 4 h before the next dose of BH_4 was administered. A Patient 1 responded well to doses as low as 5 mg/kg per day of BH_4 (*black columns, right-sided scale*). B In contrast, patient 2 showed no response to a 5 mg/kg per day dose of BH_4 but had low and stable blood phenylalanine levels at a dose of 10 mg/kg per day

concentration. Either the total amount of PAH could be increased, thereby increasing the absolute amount of active enzyme or the total amount of PAH keeps constant but the overall activity of the enzyme is ameliorated. There is no experimental evidence for a transcriptional or translational increase in PAH synthesis or a direct inhibition of PAH degrading proteases. However, we consider it most likely that interindividual differences in cellular handling of PAH folding mutants will contribute to the observed phenotypic variability and may modulate the responsiveness to BH_4 activation.

Since one of our patients (patient 2) showed a rather moderate response to BH_4, we consider a 24 h phenylalanine determination after the first BH_4 administration helpful to detect slow-responding individuals. As a consequence of our observations, we recommend to determine individually the oral BH_4 dose necessary to maintain the blood phenylalanine in the desired range, as our patients required quite divergent BH_4 doses during treatment, ranging from 5 to 20 mg/kg and day. Our results indicate the feasibility of a BH_4 monotherapy in selected patients with phenylketonuria. Furthermore, evidence from our and previous studies substantiates the role of additional factors like chaperones in the phenotypic expression of genetic diseases.

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References

1. Erlandsen H, Stevens RC (2001) A structural hypothesis for BH_4 responsiveness in patients with mild forms of hyperphylalaninaemia and phenylketonuria. J Inherit Metab Dis 24: 213–230
2. Guldberg P, Rey F, Zschocke J, Romano V, Francois B, Michiels L, Ullrich K, Hoffmann G, Burgard P, Schmidt H, Meli C, Riva E, Dianzani I, Ponzone A, Rey J, Güttler F (1998) A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype–based prediction of metabolic phenotype. Am J Hum Genet 63: 71–79
3. Kayaalp E, Treacy E, Waters PJ, Byck S, Nowacki P, Scriver CR (1997) Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a metanalysis of genotype–phenotype correlations. Am J Hum Genet 61: 1309–1317
4. Kure S, Hou DC, Ohura T, Iwamoto H, Suzuki S, Sugiyama N, Sakamoto O, Fujii K, Matsubara Y, Narisawa K (1999) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. J Pediatr 135: 375–378
5. Lindner M, Haas D, Zschocke J, Burgard P (2001) Tetrahydrobiopterin responsiveness in phenylketonuria differs between patients with the same genotype. Mol Genet Metab 73: 104–106
6. Spaapen LJM, Bakker JA, Velter C, Loots W, Rubio-Gonzalbo ME, Forget PP, Dorland L, De Koning TJ, Poll-The BT, Ploos van Amstel HK, Bekhof J, Blau N, Duran M (2001) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency in Dutch neonates. J Inherit Metab Dis 24: 352–358

Friedrich K. Trefz · Christa Aulela-Scholz · Nenad Blau

Successful treatment of phenylketonuria with tetrahydrobiopterin

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Sir: Worldwide newborn screening for phenylketonuria (PKU) and early dietary treatment of patients with impairment of the enzyme phenylalanine hydroxylase has resulted in prevention of mental retardation in more than 3000 patients [3]. However, a small number of patients with increased blood phenylalanine (Phe) levels have a defect of the coenzyme tetrahydrobiopterin (BH_4) which leads to hyperphenylalaninaemia (HPA) and neurotransmitter deficiency. They are treated by BH_4 and neurotransmitter supplementation [1]. Up to now, no patient with a defect in the apoenzyme was found who can simply be treated by supplementation of BH_4. One of our PKU patients responsive to BH_4 supplementation was found in the newborn screening programme with blood Phe levels of 96 μmol/l (reference range 36–108 μmol/l) and at 14 days of age of 885 μmol/l. BH_4 loading (20 mg/kg body weight) resulted in a decrease of blood Phe to 67 μmol/l 8 h post-loading. Under normal feeding with a breast milk adapted formula, plasma Phe levels rose again to 934 μmol/l. With a daily supplementation of 10 mg/kg of BH_4 (Dr. Schircks Laboratories, Jona, Switzerland), blood Phe levels dropped again and remained between 84 and 222 μmol/l. Surprisingly, there was no BH_4 coenzyme deficiency (normal values for neopterin and biopterin in urine, normal dihydropteridine reductase activity in red blood cells, and normal neurotransmitters and pterins in cerebrospinal fluid). However, mutation analysis of the phenylalanine hydroxylase gene revealed the two mutations IVS10G^{-11}A in intron 10 and E390G in exon 11. The first one creates a zero activity of the enzyme, the second one is a missense mutation, together resulting in a phenotype with mild PKU [4]. The patient is now at 10 months of age on 10 mg BH_4/kg per day and developing normally. We speculate that there may be more mutations resulting in a K_m-variant of the phenylalanine hydroxylase enzyme in which enhancement of the residual activity can be achieved by supplementation of BH_4 as recently also found in hyperphenylalaninaemic patients in Japan [2]. Our observation strongly emphasises the necessity of the BH_4 loading test in the newborn period and further DNA mutation analysis in hyperphenylalaninaemic patients responsive to BH_4 supplementation. BH_4 supplementation instead of a low Phe dietary treatment may be possible in at least some patients with PKU or mild PKU. In these cases, treatment compliance with coenzyme substitution may be much better in adulthood.

References

1. Blau N, Thöny B, Cotton RGH, Hyland K (2001) Disorders of tetrahydrobiopterin and related biogenic amines. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Vogelstein B (eds) The metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, pp 1275–1776
2. Kure S, Hou DC, Ohura T, Iwamoto H, Suzuki S, Sugiyama N, Sakamoto O, Fujii K, Matsubara Y, Narisawa K (1999) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. J Pediatr 135: 375–378
3. Schweitzer-Krantz S, Burgard P (2000) Survey of national guidelines for the treatment of phenylketonuria. Eur J Pediatr 159[Suppl 2]: S70–S73
4. Scriver CR, Waters PJ, Sarkissian C, Ryan S, Prevost L, Cote D, Novak J, Teebi S, Nowacki PM (2000) *PAH*db: a locus-specific knowledgebase. Hum Mutat 15: 99–104

F. K. Trefz (✉)
Klinik für Kinder und Jugendmedizin
Reutlingen, School of Medicine,
University of Tübingen,
Steinbergstrasse 31,
72764 Reutlingen, Germany
Tel.: +49-7121-939934
Fax: +49-7121-939935

C. Aulela-Scholz
Institute of Clinical Genetics,
School of Medicine,
University of Tübingen, Germany

N. Blau
Division of Clinical Chemistry
and Biochemistry,
University Children's Hospital,
Zürich, Switzerland

Inhalation of the Nitric Oxide Synthase Cofactor Tetrahydrobiopterin in Healthy Volunteers

ROLAND WALTER, NENAD BLAU, ANDREAS SCHAFFNER, MARKUS SCHNEEMANN, RUDOLF SPEICH, RETO STOCKER, BÄRBEL NAUJECK, and GABRIELE SCHOEDON

Departments of Medicine and Surgery, University Hospital; and Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zürich, Switzerland

Pulmonary endothelial dysfunction is the hallmark of acute lung injury. Impaired pulmonary endothelial nitric oxide (NO) production in this event has been described. Tetrahydrobiopterin (BH_4) is an essential cofactor for NO synthase and modulator of its activity. At high local concentrations, BH_4 provokes local vasodilation *in vivo* in healthy individuals. At lower concentrations, BH_4 selectively and locally restores disturbed NO-dependent vasodilation in patients with endothelial dysfunction. In this preliminary study, we therefore investigated the feasibility of BH_4 inhalation in five healthy human volunteers. Inhalation of buffered, aqueous BH_4-dihydrochloride solution was well tolerated; despite the buffer, BH_4 stability was completely preserved. Resorption of inhaled BH_4 was demonstrated by significantly increased BH_4 levels in plasma and urine. Inhaled BH_4 did not alter pulmonary function and had no effect on systemic hemodynamic values. Our data demonstrate that inhalation is a novel method for local BH_4 administration, offering a basic therapeutic tool for investigation of restoration of impaired NO-dependent vasodilation due to pulmonary endothelial dysfunction. Walter R, Blau N, Schaffner A, Schneemann M, Speich R, Stocker R, Naujeck B, Schoedon G. Inhalation of the nitric oxide synthase cofactor tetrahydrobiopterin in healthy volunteers.

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Pulmonary endothelial dysfunction is a major event in acute lung injury, and impaired endothelial production of nitric oxide (NO) has been described in the early stages of vascular disorders (1). Recently, evidence has emerged suggesting that the capacity of pulmonary endothelial cells to produce endogenous NO is impaired in acute lung injury (1). Constitutive NO synthase (cNOS) and inducible NOS (iNOS) have been localized within lung tissue (2–4), but the regulation of NOS expression in physiologic and pathologic states in the lung remains poorly understood.

The activity of cNOS and iNOS is modulated by the cofactor tetrahydrobiopterin (BH_4) (5, 6). De novo biosynthesis of BH_4 from GTP is highly regulated (7, 8). Endogenous BH_4 is insufficient for full iNOS activity in rat smooth muscle cells (9, 10) and exogenous BH_4 regulates iNOS expression by stabilization of its mRNA (11). Exogenous BH_4 induces relaxation of isolated rat and canine arteries (12, 13). Reconstitution of endothelium-dependent vasodilation after reperfusion injury by BH_4 has been demonstrated in a pig model (14). In man, BH_4 intra-arterially infused in relatively high concentrations has marked local vasodilating properties in healthy subjects but does not affect systemic blood pressure or heart rate (15). Moreover, BH_4 intraarterially infused in a relatively low concentration selectively and locally restores NO-dependent vasodilation in patients with endothelial dysfunction due to hypercholesterolemia, while it has no effect in healthy control subjects (16). These results suggest that impaired endogenous BH_4 synthesis contributes to disturbed NO production in the vascular system.

Supplementation of the impaired endogenous NO production by inhalational NO has been shown to be beneficial for treatment of patients with acute or chronic pulmonary diseases (17, 18). However, questions remain concerning the side effects as well as the practical aspects of inhalational NO, and strict guidelines must be followed for its safe administration (17). Thus, it is of interest to search for additional pharmacological approaches aimed at restoring NO dependent vasodilation in the lung. Inhalation of the NO synthase cofactor BH_4 might be such a therapeutic modality.

Whether BH_4 could affect NO dependent lung dysfunction is still unknown. Therefore, the purpose of this preliminary report is to investigate whether BH_4 can be administered by inhalation in humans, whether inhaled BH_4 is locally resorbed and whether inhaled BH_4 exerts any adverse effect on pulmonary function or systemic hemodynamic parameters in healthy individuals.

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Correspondence and requests for reprints should be addressed to Dr. G. Schoedon, Department of Medicine, University Hospital, CH-8091 Zürich, Switzerland. E-mail: klinsog@usz.unizh.ch

METHODS

Study Population

The pilot test with BH_4 dissolved in N-acetylcysteine was performed on one subject. Inhalation experiments with buffered BH_4 were performed on four subjects. All subjects (four male, one female; age 30–46 yr) were in good health and had no history of lung disease. The study was performed according to the standards of the local ethics committee. Blood samples were drawn from the A. radialis and V. brachialis before and immediately after completing the inhalation in heparinized tubes (Vacutainer; Becton Dickinson, Rutherford, NJ). Plasma was separated and kept frozen at −20° C. Midstream urine was collected in sterile tubes and immediately frozen at −20° C.

Determination of BH_4 Stability

Three hundred mg of BH_4-dihydrochloride were dissolved in 3 ml sterile water. The clear and colorless solution was titrated with $NaHCO_3$ to a pH of 3.0, 3.8, 4.5, and 5.0, respectively, and strongly stirred at room temperature in air atmosphere and dim light. After 30 min, the maximal time needed for inhalation, the color was noted and pterins were quantified by HPLC.

Administration of BH_4

For the pilot experiment, (6R)-5,6,7,8-tetrahydro-L-biopterin-dihydrochloride (Dr. B. Schircks Laboratories, Jona, Switzerland) was dissolved in N-acetylcysteine 10% (Inpharzame, Cadempino, Switzerland). N-acetylcysteine is used for galenic stabilization of BH_4 tablets (Milupa, Friedrichsdorf, Germany) for long-term storage at room temperature. Inhaled N-acetylcysteine has no known effect on lung function per se, it has, however, a bad taste. Therefore, in all other experiments, 500 mg BH_4 were dissolved, directly prior to inhalation in dim light, in 3 ml sterile water containing 150 mg $NaHCO_3$, resulting in a buffered solution with a pH of 4.5–5. For inhalation, the hand-activated DeVilbiss technique was used (19). The DeVilbiss 646 nebulizer was run with compressed dry medical air at a flow rate of 8 L/min. This technique results in an aerosol mass median diameter of 4.4 μm (range 3.7–5.8 μm) and in 57% of respirable droplets (< 5 μm) (20). The mouthpiece of the nebulizer was placed between the teeth of the subject, who was directed to exhale to functional residual capacity, inhale slowly over 1–2 s toward total lung capacity, and then hold his or her breath for 2–3 s. Throughout the inhalation, the subject activated the nebulizer by placing a finger over the activator valve. This procedure was repeated until all of the solution was nebulized. The subjects completed the inhalation within 20 to 30 min.

Pulmonary Function Measurements

Spirometry, lung volumes, resistance, and diffusion capacity for CO were measured using the body plethysmograph Sensor Medics 66200 Autobox® (Yorba Linda, CA), which satisfies the American Thoracic Society performance criteria (21). Criteria for acceptability, reproducibility and predicted values were according to the European Community for Steel and Coal (22, 23).

Hemodynamic Parameters

The heart rate and systolic, diastolic and mean arterial blood pressure were measured every 2.5 min with a Colin BP-306 blood pressure monitor (Carbamed, Liebefeld, Switzerland). Data were collected from 15 min before to 15 min after the inhalation procedure.

Pterin Measurements

Pterins in plasma and urine were quantified with fluorimetric detection by HPLC after acidic or differential oxidation as previously described (5, 24). For stability studies, BH_4 solution was analyzed directly without prior oxidation to determine spontaneous oxidation in solution.

RESULTS

Pilot Experiment

This experiment was performed on one volunteer. Since BH_4 is administered orally in a dose up to 20 mg/kg body weight (25), we considered it safe to start inhalation with 200 mg BH_4 dissolved in 3 ml N-acetylcysteine 10%. Immediately after inhalation, venous plasma levels of BH_4, detected as biopterin by HPLC, increased from 22.4 to 130.4 nmol/L. In a second step, we applied 800 mg BH_4 dissolved in 5 ml N-acetylcysteine 10% within the same experiment. Venous plasma BH_4 accordingly increased further to 599.8 nmol/L. A similar increase

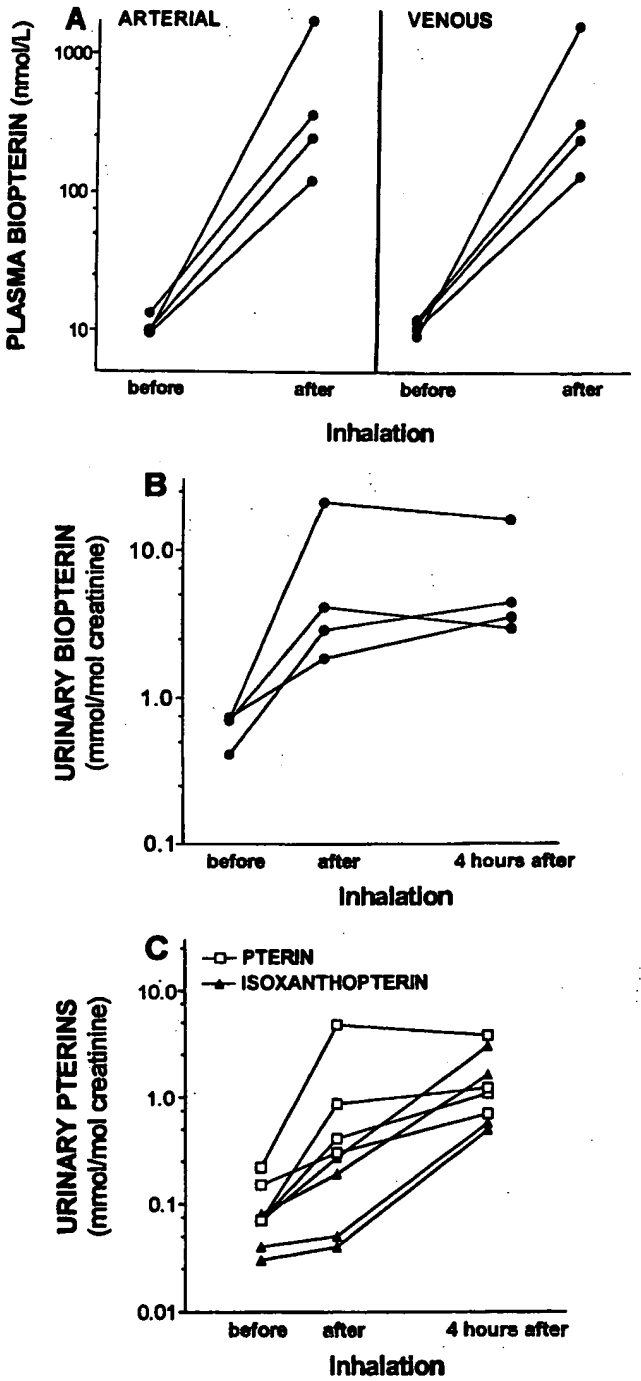

Figure 1. Alteration of plasma (*A*) and urinary (*B, C*) pterin concentrations by inhalation of a buffered BH_4 solution. Five hundred mg BH_4 were dissolved in 3 ml sterile water containing 150 mg $NaHCO_3$ just before inhalation. Blood samples were drawn before and immediately after the inhalation. Midstream urine was collected before, immediately after and 4 h after the end of inhalation. Results of experiments, performed on four individuals, are presented on a logarithmic scale.

LUNG FUNCTION BEFORE AND AFTER INHALATION OF BH_4

	Pre-inhalation (% of predicted)	Post-inhalation (% of predicted)
FVC	111.5 (88–116)	110.0 (85–118)
FEV_1	100.0 (81–109)	101.0 (76–106)
FEV_1/FVC	94.0 (92–96)	94.0 (91–96)
VC	108.0 (86–114)	108.5 (82–119)
TLC	103.0 (87–117)	102.5 (80–115)
RV	97.5 (86–115)	92.5 (81–108)
D_{LCO}	95.5 (79–116)	94.0 (74–119)

Definition of abbreviations: FVC = forced expiratory vital capacity; FEV_1 = forced expiratory volume in one second; FEV_1/FVC = FEV_1 as a percentage of FVC; VC = vital capacity; TLC = total lung capacity; RV = residual volume; D_{LCO} = diffusing capacity for carbon monoxide.

Results are given as median values (range) of the percentage of predicted.

of BH_4 was observed in the arterial blood sample, where biopterin concentrations increased from 72.1 to 548.9 nmol/L. Midstream urine was collected prior to experimentation, immediately after and 4 h after the end of the second step. Urinary BH_4 excretion increased from 0.49 to 1.14 and further to 8.37 mmol/mol creatinine. Respiratory functions, measured as spirometry, lung volumes, pulmonary resistance and diffusing capacity for CO were normal and were not altered by inhaled BH_4 (data not shown). However, BH_4 dissolved in N-acetylcysteine has an unpleasant odor and sour taste. Therefore, we decided to use buffered BH_4 solution for further inhalation studies.

Stability of Buffered BH_4

Testing of buffered BH_4 for stability was done as described in the METHODS section. Irrespective of the pH, titrated with $NaHCO_3$ to 3.0, 3.8, 4.5, or 5.0, respectively, more than 95% of the initially dissolved BH_4 was detected as unoxidized BH_4 by HPLC after vigorous stirring for 30 min in air. As the solution was stirred, its color changed from colorless to slight yellow but remained clear and odorless.

Inhalation of Buffered BH_4

The next series of experiments was performed on four further volunteers. Just before inhalation, BH_4 solution buffered to a pH of 4.5–5 was freshly prepared in dim light in a total volume of 3 ml. This solution was color- and odorless and had a slight sour taste. Directly after the inhalation period (20 to 30 min), biopterin levels were increased 13–160-fold in arterial as well as in venous plasma (Figure 1A). Similar results were observed in urine, where biopterin increased 5–25 fold (Figure 1B). The renally excreted degradation products of BH_4, pterin and isoxanthopterin (*see* Figure 3), rose accordingly (Figure 1C). However, differential oxidation of the urinary samples revealed that the percentage of BH_4 from total excreted biopterin remained in the physiologic range (60–80% tetrahydro form), indicating that a major part of circulating BH_4 after inhalation remained in the reduced form. There were no significant effects of inhaled BH_4 on respiratory function (Table 1), and buffered BH_4 was well tolerated by all subjects. The stability of buffered BH_4 solution was verified by HPLC determination of BH_4 concentrations in samples taken from the nebulizer shortly before the end of inhalation and was greater than 95% throughout.

Hemodynamic Parameters

As shown in Figure 2A–C, the systemic hemodynamic parameters were unaffected during and after inhalation of 500 mg BH_4. None of the volunteers experienced orthostatic hypotension during or after the experiment.

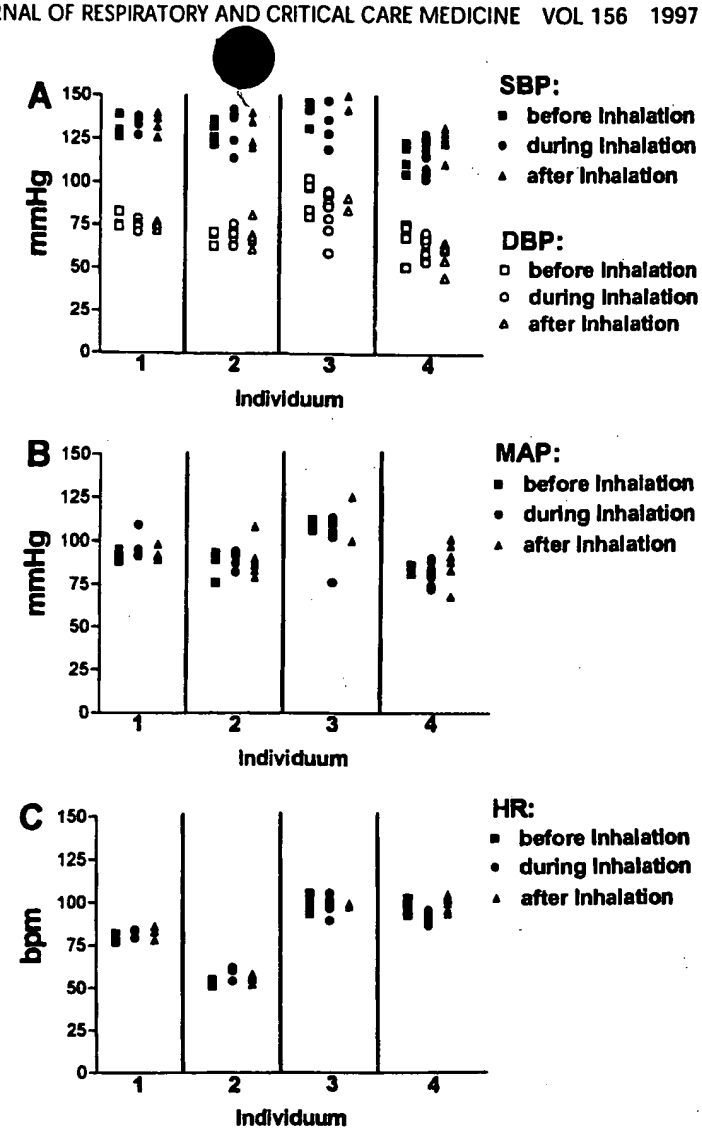

Figure 2. Hemodynamic parameters before, during and after BH_4 inhalation. Systolic and diastolic blood pressure (*A*), mean arterial pressure (*B*) and heart rate (*C*) were measured every 2.5 min before (*squares*), during (*circles*), and after (*triangles*) inhalation of 500 mg BH_4. Data were collected from 15 min before to 15 min after the inhalation procedure. The results from four different subjects of one representative experiment are presented. SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; HR = heart rate; bpm = beats per minute.

DISCUSSION

This preliminary study demonstrates: (*1*) the feasibility of BH_4 inhalation; (*2*) the local resorption of BH_4 in the lung; and (*3*) the tolerability of inhaled BH_4. There is no acute effect, and in particular no adverse effect, on pulmonary function parameters. BH_4 inhalation therefore represents a novel modality of local BH_4 administration. Our studies indicate that freshly prepared, sodium bicarbonate buffered, BH_4 solutions are stable for 30 min (the maximal time needed for inhalation of 500 mg BH_4) at a pH ranging from 3.0 to 5.0. Therefore, the pH chosen for the solution used for inhalation in this study is 4.5–5. This value is comparable to the pH of the N-acetylcysteine 10% solution routinely used for inhalation for many years. Therefore, we believe that inhalation of a solution buffered to a pH of 4.5–5 is likely to be safe, even for long-term applications. Fur-

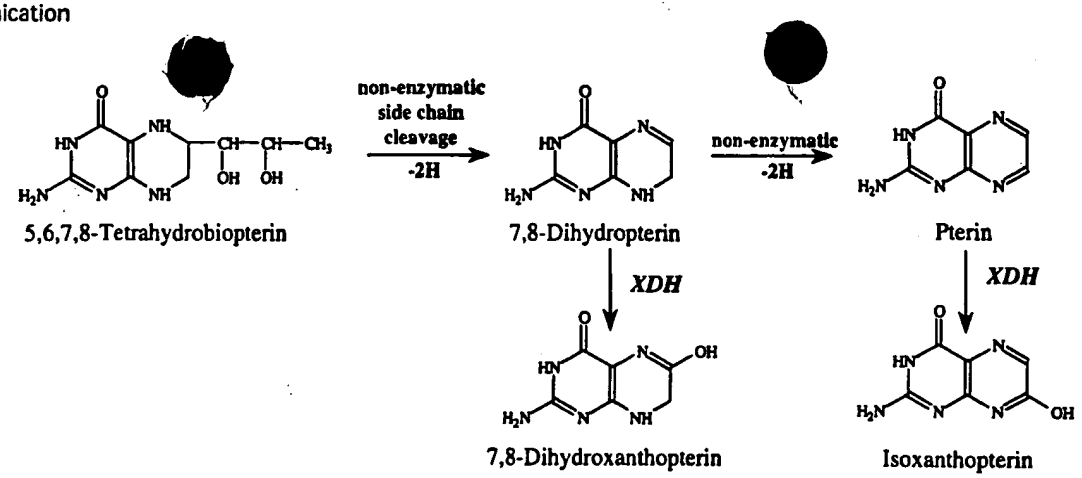

Figure 3. Metabolic breakdown of BH_4. BH_4 has a half-life of approximately 7 h in serum and over 80% of the total amount circulates in the fully reduced form (27). If degraded, it is either chemically cleaved to pterin or further enzymatically metabolized by xanthine dehydrogenase (XDH) to isoxanthopterin in the liver. Both of the degradation products, pterin and isoxanthopterin, are renally excreted and can be analyzed in urine.

thermore, our experience with orally administered BH_4, used for life-long daily therapy of inborn BH_4 deficiencies for over 15 years in doses comparable to that used for inhalation, reveals no adverse side-effects or toxicity.

The vasodilating properties of BH_4 have been shown *in vitro* and *in vivo* (12–16). One great advantage of inhaled NO in the treatment of pulmonary diseases is its selective pulmonary vasodilation without major influence on the systemic circulation, explained by the inactivation of NO by rapid combination with hemoglobin in the pulmonary circulation (26). Our data show that systemic hemodynamics are not affected by inhalation of 500 mg BH_4 despite elevated systemic arterial and venous BH_4 levels. Elevated urinary excretion of pterin and isoxanthopterin, the breakdown products of BH_4 exclusively, but not of more highly oxidized biopterins (*see* Figure 3), indicate that the circulating substance was in the fully reduced form. Infusion of high doses of BH_4 (8–32 mg/min) in the brachial artery in man led to a marked local vasodilation in the perfused limb. Blood flow in the nonperfused limb, systemic blood pressure and heart rate remained unchanged despite elevated circulating BH_4 concentrations (15). These results suggest a requirement of high local BH_4 concentrations for vasodilating effects of exogenous BH_4 in healthy subjects. Most interestingly, a relatively low dose of BH_4 (500 μg/min) locally restored the disturbed NO-dependent vasodilation of patients with endothelial dysfunction due to hypercholesterolemia, while the same concentration of BH_4 had no effect in control subjects (16). Thus, local administration of lower doses of BH_4 might be a therapeutic approach for selective restoration of impaired endothelial NO production leading to vasodilation, and local administration of BH_4 by inhalation might therefore be advantageous to intravenous BH_4. Furthermore, inhaled BH_4 should be distributed predominantly to well ventilated alveoli, thereby improving the matching of ventilation to perfusion, resulting in improved arterial oxygenation.

In summary, our preliminary study in five healthy subjects shows that BH_4 inhalation is a feasible modality for local application of this regulatory NO synthase cofactor in the lung. It remains to be established whether BH_4 restores the impaired pulmonary endothelial cell NO production in patients with NO dependent pulmonary diseases and might thereby offer an alternative therapeutic approach aimed at restoration of NO dependent vasodilation in the lung.

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References

1. Rimar, S., and C. N. Gillis. 1997. Nitric oxide and experimental lung injury. *In* W. M. Zapol and K. D. Bloch, editors. Nitric Oxide and the Lung. Marcel Dekker, New York, Basel, Hong Kong. 165–183.
2. Guo, F. H., H. R. De Raeve, T. W. Rice, D. J. Stuehr, F. B. J. M. Thunnissen, and S. C. Erzurum. 1995. Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 92:7809–7813.
3. Asano, K., C. B. E. Chee, B. Gaston, C. M. Lilly, C. Gerard, J. M. Drazen, and J. S. Stamler. 1994. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 91:10089–10093.
4. Giaid, A., and D. Saleh. 1995. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* 333:214–221.
5. Schoedon, G., M. Schneemann, S. Hofer, L. Guerrero, N. Blau, and A. Schaffner. 1993. Regulation of the L-arginine-dependent and tetrahydrobiopterin-dependent biosynthesis of nitric oxide in murine macrophages. *Eur. J. Biochem.* 213:833–839.
6. Rosenkranz-Weiss, P., W. C. Sessa, S. Milstien, S. Kaufman, C. A. Watson, and J. S. Pober. 1994. Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells: elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. *J. Clin. Invest.* 93:2236–2243.
7. Schoedon, G., M. Schneemann, N. Blau, C.-J. S. Edgell, and A. Schaffner. 1993. Modulation of human endothelial cell tetrahydrobiopterin synthesis by activating and deactivating cytokines: new perspectives on endothelium-derived relaxing factor. *Biochem. Biophys. Res. Commun.* 196:1343–1348.
8. Di Silvio, M., D. A. Geller, S. S. Gross, A. Nussler, P. Freeswick, R. L. Simmons, and T. R. Billiar. 1993. Inducible nitric oxide synthase activity in hepatocytes is dependent on the coinduction of tetrahydrobiopterin synthesis. *Adv. Exp. Med. Biol.* 338:305–308.
9. Gross, S. S., and R. Levi. 1992. Tetrahydrobiopterin synthesis: an absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J. Biol. Chem.* 267:25722–25729.
10. Walter, R., N. Blau, L. Kierat, A. Schaffner, and G. Schoedon. 1996. Effects of activating and deactivating cytokines on the functionally linked tetrahydrobiopterin - NO pathways in vascular smooth muscle cells. *Immunol. Lett.* 54:25–29.
11. Saura, M., D. Pérez-Sala, F. J. Cañada, and S. Lamas. 1996. Role of tetrahydrobiopterin availability in the regulation of nitric-oxide synthase expression in human mesangial cells. *J. Biol. Chem.* 271:14290–14295.
12. Van Amsterdam, J. G. C., and J. Wemer. 1992. Tetrahydrobiopterin induces vasodilatation via enhancement of cGMP level. *Eur. J. Pharmacol.* 215:349–350.

13. Cosentino, F., and Z. S Katušic. Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. *Circulation* 91:139–144.
14. Tiefenbacher, C. P., W. M. Chilian, M. Mitchell, and D. V. Defily. 1996. Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. *Circulation* 94:1423–1429.
15. Schaffner, A., N. Blau, M. Schneemann, J. Steurer, C.-J. S. Edgell, and G. Schoedon. 1994. Tetrahydrobiopterin as another EDRF in man. *Biochem. Biophys. Res. Commun.* 205:516–523.
16. Stroes, E., J. Kastelein, F. Cosentino, W. Erkelens, R. Wever, H. Koomans, T. Lüscher, and T. Rabelink. 1997. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J. Clin. Invest.* 99:41–46.
17. Zapol, W. M., S. Rimar, N. Gillis, M. Marletta, and C. H. Bosken. 1994. Nitric oxide and the lung. *Am. J. Respir. Crit. Care Med.* 149:1375–1380.
18. Cornfield, D. N., and S. H. Abman. 1996. Inhalation nitric oxide in pulmonary parenchymal and vascular disease. *J. Lab. Clin. Med.* 127:530–539.
19. Yan, K., C. Salome, and A. J. Woolcock. 1983. Rapid method for measurement of bronchial responsiveness. *Thorax* 38:760–765.
20. Newman, S. P., P. G. Pellow, and S. W. Clarke. 1986. Droplet size distributions of nebulised aerosols for inhalation therapy. *Clin. Phys. Physiol. Meas.* 7:139–146.
21. Standardization of spirometry—1987 update: Statement of the American Thoracic Society, 1987. *Am. Rev. Respir. Dis.* 136:1285–1298.
22. Quanjer, P. H., G. J. Tammeling, J. E. Cotes, O. F. Pedersen, R. Peslin, and J. C. Yernault. 1993. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur. Respir. J. Suppl.* 16:5–40.
23. Cotes, J. E., D. J. Chinn, P. H. Quanjer, J. Roca, and J. C. Yernault. 1993. Standardization of the measurement of transfer factor (diffusing capacity). Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur. Respir. J. Suppl.* 16:41–52.
24. Curtius, H. C., N. Blau, and T. Kuster. 1991. Pterins. *In* F. A. Hommes, editor. Techniques in Diagnostic Human Biochemical Genetics. Wiley-Liss, New York. 377–396.
25. Ponzone, A., O. Guardamagna, M. Spada, S. Ferraris, R. Ponzone, L. Kierat, and N. Blau. 1993. Differential diagnosis of hyperphenylalaninaemia by a combined phenylalanine-tetrahydrobiopterin loading test. *Eur. J. Pediatr.* 152:655–661.
26. Rimar, S., and C. N. Gillis. 1993. Selective pulmonary vasodilation by inhaled nitric oxide is due to hemoglobin inactivation. *Circulation* 88: 2884–2887.
27. Ponzone, A., O. Guardamagna, M. Spada, R. Ponzone, M. Sartore, L. Kierat, C. W. Heizmann, and N. Blau. 1993. Hyperphenylalaninemia and pterin metabolism in serum and erythrocytes. *Clin. Chim. Acta* 216:63–71.

EXHIBIT X

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SHORT REPORT

Tetrahydrobiopterin responsiveness in a large series of phenylketonuria patients

J. Weglage, M. Grenzebach, A. v. Teeffelen-Heithoff, T. Marquardt, R. Feldmann, J. Denecke, D. Gödde and H. G. Koch*

Department of Pediatrics, University Hospital of Münster, Germany

*Correspondence: Department of Pediatrics, University Hospital of Münster, Albert-Schweitzer-Str. 33, D-48129 Münster, Germany. E-mail: weglage@uni-muenster.de

Sumary: In a group of 87 consecutive patients with hyperphenylalaninaemia born since 1990, only 3 patients showed a (temporary) decrease of serum phenylalanine levels after tetrahydrobiopterin (BH_4) loading in usual doses (20 mg/kg body weight).

Kure and colleagues (1999) reported the first identification of a tetrahydrobiopterin (BH_4)-responsive phenylalanine hydroxylase (PAH) deficiency in four patients with a positive PKU screening result (phenylalanine >120 μmol/L). Most recently, Lindner and colleagues (2001) described three patients with PAH deficiency and the same genotypes but different responses to standardized BH_4 loading. They concluded that BH_4 responsiveness in PAH deficiency is at least partly independent of PAH genotype. Trefz and colleagues (2001) reported a successful treatment with BH_4 (10 mg/kg body weight) in one patient with mild PKU and concluded that BH_4 supplementation instead of a phenylalanine-restricted diet might be possible in at least some patients with classical or mild PKU. The prevalence of BH_4 responsiveness in PAH deficiency is still unclear. We therefore retrospectively evaluated results of the BH_4 loading test in the newborn period of our 87 patients born between 1990 and 2001.

The loading test was routinely performed in all patients with a positive newborn screening (phenylalanine >120 μmol/L) with 20 mg/kg body weight BH_4 (Dr Schircks Laboratories, Jona, Switzerland), continuing a normal protein diet. Serum phenylalanine levels were measured by HPLC before and 4 h and 8 h after BH_4 intake. A BH_4 coenzyme deficiency was excluded in all patients (normal values for neopterin and biopterin in urine, normal dihydropterin reductase activity in red blood cells, measured by N. Blau, Zürich, Switzerland). A 50% decrease of the serum phenylalanine level after BH_4 intake was defined as a significant decrease as in all reported BH_4-responsive patients after BH_4 supplementation. Pre-loading serum phenylalanine (Phe) levels were within a wide range (132–3036 μmol/L). Three of the 87 patients (3.5%) showed a significant decrease of serum phenylalanine concentrations 8 h after BH_4 intake (84 patients, 96.5% did not). Theses three patients will be described in more detail.

Patient 1: A boy, born 11/99, normal pregnancy and birth. Phe levels: 678 μmol/L in the screening test; 864 μmol/L before, 684 μmol/L 4 h and 252 μmol/L 8 h after BH_4 loading. On free nutrition supplemented with 10 mg/kg body weight BH_4 per day Phe levels increased within 6 weeks to 972 μmol/L. BH_4 treatment was stopped and a Phe-restricted diet was started. Genotype: IVS3–22G>A/Y414C.

Patient 2: A boy, born 11/00, normal pregnancy and birth. Phe levels: 360 μmol/L in the screening test; 396 μmol/L before, 192 μmol/L 4 h, and 90 μmol/L 8 h after BH_4 loading. On free nutrition (without BH_4 Phe levels increased to 642 μmol/L. On free nutrition supplemented with 10 mg/kg body weight BH_4 per day Phe levels were stable between 540 and 660 μmol/L. BH_4 treatment was stopped and a protein-restricted diet was started. DNA for genotyping not available.

Patient 3: A girl, born 12/00, normal pregnancy and birth. Phe levels: 978 μmol/L in the screening test; 1032 μmol/L before, 702 μmol/L 4 h and 432 μmol/L 8 h after BH_4 loading. On free nutrition supplemented with 10 mg/kg body weight BH_4 per day Phe levels persisted around 600 μmol/L. Continuing BH_4 treatment, the initiation of a protein-restricted diet (450 mg Phe/day) was neccessary to achieve satisfactory metabolic control. Under this treatment Phe levels were measured in a range of 300–420 μmol/L. A protein-restricted diet without BH_4 will be tested. Genotype: Y414C/A104D.

Recently, an alternative treatment of at least a subgroup of patients with a PAH deficiency with BH_4 supplementation has been discussed (Kure et al 1999; Trefz et al 2001; Lindner et al 2001). In our group of 87 consecutive patients born since 1990, only 3 patients showed a significant decrease of Phe levels after BH_4 loading. In none of the patients did BH_4 treatment prove to be an effective therapy.

In conclusion, BH_4 supplementation in usual doses might be an alternative treatment in rare cases of PAH deficiency. However, even a positive BH_4 loading test in the newborn period does not suggest BH_4-responsive phenylketonuria in the long term. It is still unclear why phenylalanine levels were rising in our initial BH_4-responsive patients in spite of ongoing BH_4 treatment.

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REFERENCES

Kure S, Dian-Chang H, Toshibiro O, et al (1999) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *J. Pediatrics* **135**: 375–378.

Lindner M, Haas D, Zschocke J, et al (2001) Tetrahydrobiopterin responsiveness in phenylketonuria differs between patients with the same genotype. *Mol Genet Metab* **73**(1): 104–106.

Trefz K, Aulela-Scholz C, Blau N (2001) Successful treatment of phenylketonuria with tetrahydrobiopterin. *Eur J Pediatr* **160**(5): 315.